

STUDIES OF METAL - ORGANIC INTERACTIONS
WITH MODEL SYNTHETIC AND NATURAL
LIGANDS APPLICABLE TO NATURAL WATERS

THESIS BY
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IN PARTIAL FULFILLMENT OF THE REQUIREMENTS
FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY

CALIFORNIA INSTITUTE OF TECHNOLOGY
PASADENA, CALIFORNIA

2001

(SUBMITTED NOVEMBER 17, 2000)

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Acknowledgements

I would like to thank my advisor, Janet Hering, for everything I have learned from her, for her tremendous input into this work and for her fruitless efforts to teach me technical writing in English.

I am grateful to Prof. A. J. Colussi and Prof. Jim Morgan for their help with this project and for their support and encouragement. I also would like to thank Nathan Dalleska and Dr. Suzanna Horvath for their help with the phytochelatin studies, Angel Di Bilio for the EPR analysis, Peter Green, Bob Becker, and Yaniv Dubowski for their help with various analytical techniques. It has been a pleasure to share the lab with Tina Salmasi, Jennie Stephens and Dan Giammar. It was wonderful to share my Caltech experience for five years with Penny Kneebone who has helped me in many ways. I would not be able to do this work without my friends Maxim Lyutikov, Anna Kashina, Peter Michalev, Hugo Destailats, Elena Escot, Fok Leung, Olga and Anton Ivanov, Anastasia Nikitina and Oleg Kovrizhkin, Boris and Natalia Merinov. I certainly would not finish (would not even start) this work without all the support, help and encouragement of my friend Jim Gallaway. I have been very lucky with my roommates and friends Sergei Cherkis and Yuri Levin who had to share a lot of my frustrations. I am very grateful to my four-legged friends Yasha, Refund, Junior and Bonus who kept reminding me that the world is bigger than Caltech, which was not always obvious during these years.

I am especially grateful to my family, my husband, my parents and my sister for their love and understanding.

Abstract

Metal speciation significantly influences the geochemical cycling of trace metals and can control metal bioavailability and toxicity. This study addressed some unresolved questions concerning metal speciation in natural waters in several complementary ways.

Metal competition for model and natural organic ligands was examined by coupling size-exclusion chromatography (SEC) with inductively-coupled plasma mass spectrometry. The method was validated with well-defined organic ligands in ligand-competition studies with a single metal and a binary metal mixture. The measured concentrations of metal-ligand species corresponded to the calculated equilibrium speciation. However, the method is subject to kinetic limitations. For metal complexes that are partially labile during chromatographic separation, the rate constant for complex dissociation and the concentration of the (initial) complex were estimated based on a mathematical model.

Application of this method to studies of copper complexation by Suwannee River humic acid demonstrated that copper complexes are kinetically labile on the SEC column for copper additions comparable to the background concentrations in the humic acid samples. This suggests that the copper-binding sites that form complexes detectable by this method are present in humic acids at very low concentrations and are not available to bind added copper.

Investigation of copper complexation with a synthetic analog of the natural metal-binding peptide phytochelatin has demonstrated that Cu(II) is not stable in the presence

of phytochelatin but that the peptide forms strong complexes with Cu(I). Complexation of Cu(I) by phytochelatin was studied using a spectroscopic technique in which bathocuproine was added as a competing ligand. The method was validated in experiments with glutathione.

Titration of bathocuproine and phytochelatin with Cu(I) and of Cu(I) and bathocuproine with phytochelatin were explained by formation of 1:1 and 1:2 Cu(I)-phytochelatin complexes. To account for the experimental observations, a polynuclear (1:2:1) phytochelatin-Cu(I)-bathocuproine complex was introduced in modeling the titration data. Consistent values of conditional stability constants were obtained in Cu(I) and phytochelatin titrations. However these results were not consistent with those obtained in titrations of Cu(I) and phytochelatin with bathocuproine possibly due to the slow ligand exchange kinetics of the pre-formed Cu(I)-phytochelatin complexes.

Contents

Acknowledgements	iii
Abstract	iv
1 Introduction and Motivation	1-1
1.1 Importance of metal speciation	1-1
1.2 Objectives	1-3
2 Background	2-1
2.1 Analytical approaches	2-2
2.1.1 Analytical techniques based on measuring free or labile metal.. ..	2-2
2.1.2 Analytical approaches based on physical separation	2-7
2.2 Experimental observations.. .. .	2-10
2.2.1 Sulfides	2-12
2.2.2 Colloids	2-13
2.2.3 Organics	2-14
2.2.4 Modeling	2-15
2.3 Organic ligands in natural waters	2-17
2.3.1 Well-defined ligands	2-17
2.3.2 Ill-defined ligands	2-21
2.4 Concluding remarks	2-23
3 Direct quantification of metal-organic interactions by SEC and ICP-MS	3-1
3.1 Introduction	3-1

3.2	Materials and methods	3-5
3.2.1	Reagents and standard solutions	3-5
3.2.2	Instrumentation	3-6
3.2.3	Calibration and column characterization	3-8
3.2.4	Chromatographic conditions and coupling to ICP-MS	3-8
3.2.5	Equilibrium speciation calculations	3-9
3.3	Results and discussion	3-11
3.3.1	Ligand competition with a single metal	3-11
3.3.2	Ligand competition with a binary metal mixture	3-13
3.3.3	Method limitations due to complex lability during SEC	3-15
3.3.4	Method limitations due to incomplete separation of complexes by SEC	3-19
3.3.5	Implications of experimental results for previous studies	3-21
3.4	Conclusions	3-23
	Acknowledgements	3-24
	Appendix 3A	3-25
4	Mathematical modeling of the chromatographic behavior of metal complexes	4-1
4.1	Introduction	4-1
4.2	System description and model assumptions	4-5
4.2.1	Model parameters	4-5
4.2.2	Limiting behavior of metal complexes	4-11
4.3	Mathematical description of the system	4-15

4.3.1	Governing equations	4-15
4.3.2	Concentration profile for the metal complex.. .. .	4-16
4.3.2.1	Elution time for the complexed metal	4-17
4.3.3	"Free" metal concentration profile	4-18
4.3.4	Expression for the total amount of metal (neglecting diffusion) ..	4-19
4.4	Experimental determination of rate constant for dissociation and the equilibrium concentration of the metal complex.. .. .	4-23
4.5	Concluding remarks	4-29
	Acknowledgements	4-30
	Appendix 4A	4-31
5	Studies of copper-humic interactions using SEC/ICP-MS	5-1
5.1	Introduction	5-1
5.2	Background	5-2
5.2.1	Sources and composition of humics	5-2
5.2.2	Copper complexation by humic and fulvic acids	5-4
5.3	Materials and methods	5-7
5.3.1	Reagents	5-7
5.3.2	Instrumentation	5-8
5.4	Results and discussion	5-9
5.4.1	Chromatographic behavior of humic acids	5-9
5.4.2	Concentration of strong copper-binding sites in humic acids	5-12
5.4.3	Humic acid - EDDHA competition experiments	5-18
5.5	Concluding remarks	5-22

6	Investigation of copper-phytochelatin complexes using a spectroscopic technique with bathocuproine	6-1
6.1	Introduction	6-1
6.2	Background	6-3
6.2.1	Chelating functional groups	6-6
6.2.2	Oxidation state of copper in phytochelatin complexes	6-7
6.2.3	Stability of metal-phytochelatin complexes	6-8
6.2.4	Stoichiometry of Cu-phytochelatin complexes	6-9
6.4	Experimental methods	6-12
6.4.1	Reagents	6-12
6.4.2	Analytical methods	6-13
6.4.3	Titration experiments	6-13
6.4.4	Experimental data modeling	6-14
6.4.5	Estimation of Cu(I)-bathocuproine (CuB_2) conditional stability constant	6-17
6.5	Results and discussion	6-19
6.5.1	Valence state of copper ions bound to phytochelatin	6-19
6.5.1.1	Bathocuproine assay	6-20
6.5.1.2	EPR analysis	6-22
6.5.2	Investigation of Cu(I)-glutathione complexation using a spectroscopic technique with bathocuproine	6-24
6.5.2.1	Treatment of the titration data	6-27

6.5.2.2	Comparison of predicted and experimental Cu(I) speciation	6-28
6.5.2.3	Plausibility of a mixed complex.. .. .	6-28
6.5.2.4	Inclusion of a ternary (BCuG) complex in modeling experimental data	6-30
6.5.2.5	Evaluation of obtained results	6-35
6.5.2.6	Possible interference of the mixed complex	6-36
6.5.2.7	Concluding remarks	6-38
6.5.3	Investigation of Cu(I)-phytochelatin complexation using a spectroscopic technique with bathocuproine	6-39
6.5.3.1	Cu titration experiments	6-42
6.5.3.2	Phytochelatin titration experiments	6-46
6.5.3.4	Bathocuproine titration experiments	6-48
6.5.3.4	Kinetic studies of Cu(I)/P/B system	6-52
6.6	Implications of Cu-phytochelatin complexation studies	6-55
6.6.1	Sources, occurrence, and inertness of Cu(I) in natural waters	6-56
6.6.2	Thiols as strong Cu(I) binding ligands in natural waters	6-60
6.6.3	Cu(I)-thiol complexation in natural waters	6-61
6.7	Concluding remarks	6-63
	Appendix 6A	6-66
	Appendix 6B	6-71
7	Summary and conclusions	7-1
7.1	Direct quantification of metal-organic interactions by size-	

exclusion chromatography (SEC) and inductively coupled	
plasma mass spectrometry (ICP-MS)	7-2
7.2 Mathematical modeling of the chromatographic behavior of	
metal complexes	7-4
7.3 Studies of copper-humic interactions using SEC/ICP-MS	7-6
7.4 Investigation of copper-phytochelatin complexation using a	
spectroscopic technique with bathocuproine	7-8
8 References	8-1

List of Figures

3.1	Comparison of measured and calculated concentrations of metal-EDTA complexes.. .. .	3-14
3.2	ICP-MS chromatogram obtained for Ni – EDTA – EDDHA system	3-17
3.3	UV chromatogram obtained with SEC separation of Cu(HQS) _x and HQS and ICP-MS measurements of Cu in different fractions	3-18
3.4	Elution volumes for various compounds of comparable molecular weight as a function of their octanol-water coefficients illustrating hydrophobic interactions on TSK 3000 PW (pH = 6, I = 10 mM).. .. .	3-20
3A.1	Calibration curves of TSK GEL 3000 PW size-exclusion column with polystyrene sulfonate standards in water, 10 mM ammonium acetate, and 10 mM sodium acetate in 0.1 M NaCl	3-26
3B.1	Schematic set-up of the size-exclusion chromatography system used in the study	3-28
3B.2	Scheme for the separation of molecules on a size-exclusion column	3-29
4.1	ICP-MS chromatogram obtained for Ni-EDTA-EDDHA system	4-6
4.2	Qualitative description of the system.. .. .	4-9
4.3	Illustration of advection- and diffusion- dominated regimes of metal complex dissociation	4-12
4.4	Metal complex, "free" metal, and total metal fluxes vs. time	4-14
4.5	ICP-MS chromatogram obtained with ICP-MS for [CuNTA] ₀ = 4 μM	

eluting from a chromatographic column (TSKGEL 3000 PW), ionic strength - 10 mM (ammonium acetate), pH = 6	4-26
4.6 Dependence of CuNTA concentration eluting from a chromatographic column (TSKGEL 3000 PW) on the elution time of the peak maximum for the complex, t_{ML} (varied flow rates)	4-27
5.1 UV chromatograms of a 500 mg/L solution of HA	5-11
5.2 ICP-MS chromatogram of Cu-humic acid (CuHA) and CuEDDHA after the separation using SEC	5-13
5.3 Dependence of Cu recovery from the SEC column as measured with ICP MS in CuHA peak on the total Cu concentration	5-15
5.4 Dependence of concentration of Cu not bound with humic acids ("free" Cu) on total Cu concentration	5-17
5.5 Dependence of Cu concentration in CuEDDHA and CuHA complexes as measured by ICP-MS after the SEC separation on the concentration of total EDDHA (a) or Cu (b)	5-19
5.6 Dependence of Cu concentration in CuHA and CuEDDHA complexes as measured by ICP-MS (a) and % Cu recovery (b) on concentration of EDDHA	5-20
5.7 Modeling of Cu and EDDHA titrations using MINEQL ⁺ for log $K_{CuHA}^{cond} = 15$	5-21
6.1 Chemical structure of phytochelatin, synthetic phytochelatin used in the study and glutathione	6-2
6.2 Chemical structures of bathocuproine, phenanthroline and of the derivatives used in linear free energy relations estimation of copper(I)-bathocuproine	

stability constant	6-17
6.3 Relationship between $\text{Log } \beta_{ML2}$ and $\text{Log } K_{HL}$ for copper(I) and 1:2 complexes of phenanthroline (Phen), 2-methyl-1,10-phenanthroline (MPhen), 2,9-dimethyl-1,10-phenanthroline (DMPhen)	6-19
6.4 Absorbance at 480 nm due to formation of 1:2 Cu(I)-bathocuproine complex in the presence of phytochelatin; copper added as Cu(II)	6-21
6.5 EPR spectra of frozen solutions of copper(II) added to 1.7 mM phytochelatin (P) in 50 mM phosphate buffer, pH 6.5	6-23
6.6 Cu(I) and bathocuproine (B) titration curves of glutathione (G)	6-26
6.7 Cu(I) speciation in the presence of glutathione (G) and bathocuproine (B) ..	6-29
6.8 Cu(I) speciation in Cu(I) titrations of glutathione (G) and bathocuproine (B) obtained using FITEQL 4.0	6-31
6.9 Cu(I) speciation in bathocuproine (B) titrations of glutathione (G) obtained using FITEQL 4.0	6-34
6.10 Titration curves for phytochelatin (P)	6-41
6.11 Cu(I) titration curves of phytochelatin (P) and bathocuproine (B)	6-43
6.12 Cu(I) speciation in Cu(I) titrations of phytochelatin (P) and bathocuproine (B) obtained using FITEQL 4.0	6-45
6.13 Cu(I) speciation in phytochelatin (P) titrations of Cu(I) and bathocuproine (B)	6-47
6.14 Cu(I) speciation in bathocuproine (B) titrations of Cu(I) and phytochelatin based on the conditional stability constants obtained in Cu(I) titration experiments	6-49
6.15 Cu(I) speciation in bathocuproine titrations of Cu(I) and phytochelatin (P) ..	6-51

6.16	Dependence of absorbance measured at 480 nm on equilibration time.. ..	6-53
6.17	Cu(I) speciation in bathocuproine (B) titrations of Cu(I) and phytochelatin (P) based on the conditional stability constants obtained in Cu(I) titration experiments.. ..	6-54
6.18	A simplified model for redox cycling of copper ions in natural waters	6-57
– 6A.1	Cu concentration measured in Cu-phytochelatin peak with ICP-MS after the complex elution from the SEC column.. ..	6-69

List of Tables

3.1	Determination of metal speciation with combined chromatographic - metal detection techniques	3-4
3.2	HPLC and ICP-MS instrumental and operational parameters	3-7
3.3	Conditional stability constants of metal complexes studied	3-10
4.1	Symbols used in the text	4-8
5.1	Reported conditional stability constants and total ligand concentrations for copper titrations of humic and fulvic acids and whole water samples	5-6
5.2	Elemental analysis data for Suwannee River humic acid	5-8
5.3	ICP-MS operational parameters for samples with high concentration of humic acid	5-9
6.1	Summary of previous studies of metal-phytochelatin studies	6-4
6.2	Symbols	6-15
6.3	Protonation and stability constant of Cu(I) complexes with phenanthroline and its derivatives ($I = 0.5$) (Martell et al., 1993)	6-17
6.4	Reported values of Cu(I)-glutathione (G) stability constants ($\log \beta_{\text{CuL}_x}$ and $\log \beta_{\text{HL}_x}$, $I = 0.5$) (Österberg et al., 1979)	6-24
6.5	Conditional stability constants ($\text{pH} = 6.5$, $I = 0.05$) of Cu(I) - glutathione (G) complexes ($\log \beta_{\text{CuL}_x}^{\text{cond}}$) calculated from the reported values with Davies equation	6-25

6.6	Optimization of conditional stability constants ($\text{pH} = 6.5$, $I = 0.05$) for Cu(I) and bathocuproine (B) titrations of glutathione	6-33
6.7	Optimization of conditional stability constants ($\text{pH} = 6.5$, $I = 0.05$) of the CuG and BCuG complexes using a non-zero mixed complex extinction coefficient	6-38
6.8	Summary of experimental conditions for Cu(I) bathocuproine (B) and phytochelatin (P) titrations ($\text{pH} = 6.5$, $I = 0.05$)	6-40
6.9	Optimization of conditional stability constants ($\text{pH} = 6.5$, $I = 0.05$) for Cu(I) and phytochelatin (P) titrations.. .. .	6-48
6B	Experimental titration data not considered in the optimization of conditional stability constants	6-71

Chapter 1

INTRODUCTION AND MOTIVATION

1.1 Importance of metal speciation

The distribution of metals between their various organic and inorganic forms (i.e., their speciation) profoundly influences their ecotoxicological impacts. Both the bioavailability and mobility of metals vary significantly depending on their speciation. The partitioning of metals between dissolved and particulate phases influence their transport and reactivity. The transport characteristics of the colloidal metal fraction resemble those of the dissolved fractions but the bioavailability and reactivity of colloidal and dissolved fractions may differ significantly.

It is widely accepted that the bioavailability and toxicity of metals are not determined solely by the total dissolved metal concentration. Even for the dissolved metal fraction, many complexed forms of the metal are not directly available for uptake by biota. Thus, metal toxicity is related to the free metal ion concentration or activity rather than to the total dissolved metal concentration (Anderson and Morel, 1982; Luoma, 1983; Morel, 1986; Bruland et al., 1991; Morel and Hering, 1993 and ref. cited). This mode of metal toxicity has also been demonstrated in the field (Hare and Tessier, 1996; Ahner et al., 1997). Metal toxicity can be mitigated by complexation with organic and inorganic

ligands unless the formed complexes are lipophilic and diffuse through the cell membrane (Florence et al., 1992; Phinney and Bruland, 1994).

In recognition of importance of free metal ion concentration, studies of metal speciation in natural waters have focused on determining this parameter. With existing techniques, however, free metal ion concentrations usually can not be measured directly but are obtained through titration experiments in which natural water samples are amended with the metal of interest. The response of the analytical technique to the (added) metal concentration is then modeled (in various ways) and the model is used to predict the free metal ion concentration at the ambient total (dissolved) metal concentration. The analytical techniques used for metal titrations (primarily electrochemical methods) do not provide any direct characterization of the dominant metal species under ambient conditions. Analytical techniques that do allow characterization of metal complexes based on their physical-chemical characteristics (primarily chromatographic methods) are rarely used for metal titrations.

The ambient equilibrium conditions are necessarily violated both in metal titrations and in the chromatographic separation of metal species. In metal titrations, re-equilibration of the metal species is assumed with each metal addition even though some studies have shown that such re-equilibration is not necessarily rapid (Capodaglio et al., 1990; Hering and Morel, 1989). Conversely, when metal species are separated chromatographically, the species distribution in the original sample will be maintained only if the dominant metal species are kinetically inert. Otherwise, the metal may be redistributed among various chromatographic fractions or lost by sorption onto the chromatographic column.

The competition between metals can clearly influence the speciation of any individual metal. Although many trace metals are present in natural waters, metal complexation studies have mostly, for analytical convenience, focused on speciation of a single metal. Thus competition between metals for naturally-occurring ligands has generally been neglected.

There is strong evidence that biogenic ligands significantly influence metal complexation in natural waters (see Chapter 2 for review). However, information on the structure and properties of such ligands is limited. Further investigations are required to determine the occurrence and properties of biogenic ligands and to elucidate their role in controlling metal speciation in natural waters.

1.2 Objectives

This research addresses the unresolved questions concerning metal speciation in natural waters in several complementary ways. Metal competition between organic ligands is examined by coupling a chromatographic separation of metal-organic complexes with a metal-specific detection technique. The issue of the kinetic lability of metal-organic complexes during chromatographic separation is examined both experimentally and theoretically. Competitive ligand exchange is also coupled with spectroscopic detection to elucidate the metal-binding properties of a synthetic phytochelatin. This compound is used as a model for natural phytochelatins, a class of thiol-containing oligopeptides that are produced by plants and fungi in response to metal stress.

This study addresses the following hypotheses:

- 1). Analytical method for quantification of metal-organic complexes. The coupling of chromatographic separation with inductively coupled plasma mass spectrometry (ICP-MS) should allow the quantification of more than a single metal in fractions associated with different metal-organic complexes. The sensitivity and metal specificity of ICP-MS should allow investigations of multi-metal systems at concentrations applicable to natural waters. Quantitative characteristics of metal-organic interactions, such as complexation conditional stability constants, could be obtained with on-line metal titration experiments. The method relies on the kinetic inertness of strong metal-organic complexes and would be subject to kinetic limitations with labile complexes.
- 2). Mathematical modeling of the chromatographic behavior of metal-organic complexes. Some metal-organic complexes would be expected to be partially or completely labile under the non-equilibrium conditions imposed by the chromatographic separation. Mathematical modeling should provide a means to interpret the behavior of partially-labile complexes.
- 3). Metal complexation by humic acids. The developed method should be applicable to studies of relatively strong metal-binding sites suggested to be present in such ubiquitous ligands as humic and fulvic acids. The more abundant and weaker binding sites of humic and fulvic acids would be expected to be partially or completely labile under the non-equilibrium conditions during the chromatographic separation.
- 4). Metal complexation by phytochelatin. Phytochelatins are thiol-rich, metal-binding oligopeptides which are produced by plants and fungi. Since phytochelatins are produced in response to metal stress and form strong complexes with metals (and particularly with copper), these peptides could potentially be important in controlling copper speciation.

Investigation of the stability of these complexes should lend insight into their influence on copper speciation in natural waters.

Metal complexation in natural waters has been extensively studied. An overview of the major findings of these studies is presented in Chapter 2. Chapter 3 describes the development of a method that couples size-exclusion chromatography (SEC) with ICP-MS for the quantification of metal-organic complexes. Model studies using well-defined metal complexes of varying stability were performed to validate the method and to determine its limitations. In Chapter 4, the dynamic processes in a chromatographic column which perturb the equilibrium conditions in the original sample and alternate the distribution of metal species are described mathematically. The mathematical model is applied for complexes that are partially labile under the chromatographic conditions in order to estimate rate constants for the dissociation of the complex and the initial concentration of the complex in the sample injected onto the column. The SEC/ICP-MS method is applied to copper complexes with natural metal-binding ligands, humic acids, in Chapter 5. In Chapter 6 the interactions of copper with phytochelatin are investigated and conditional stability constants for Cu(I)-phytochelatin complexes are determined. The conclusions of this study are presented in Chapter 7.

Chapter 2

BACKGROUND

For many metals (especially Cu but also Ni, Fe, Zn, Cd, and Pb), it has been observed that a bound metal fraction constitutes a significant part of total metal concentration (Wells et al., 1998; Wells and Bruland, 1998; Capodaglio et al., 1990; Bruland, 1992; Kozelka et al., 1997; Coale and Bruland, 1988; Donat, 1995). These conclusions are based on the observed non-reactivity of ambient metals in analytical assays which detect free aquo ions or weak inorganic complexes. A similar lack of reactivity has been observed when natural water samples are titrated by addition of metals. This lack of reactivity has been attributed to metal complexation by strong organic complexes (e.g., Croot et al., 1999; Wells et al., 1998; Wells and Bruland, 1998; Kozelka and Bruland, 1998), strong inorganic complexes, especially sulfides (Luther et al., 1996; Rozan and Benoit, 1999; Rozan et al., 1999; Al-Farawati and van den Berg, 1999), and formation of colloids (Wells et al., 2000; Greenamoyer and Moran, 1997; Mackey and Zirino, 1994).

This chapter reviews the analytical techniques used for metal speciation studies in natural waters, experimental observations and their interpretation.

2.1 Analytical approaches

There are two general approaches to quantitative metal complexation studies. The first is based on direct measurements of the free metal aquo ion or of both the aquo ion and labile complexes of the metal. The second relies on chromatographic separation of metals into fractions and quantification of the metal associated with each fraction. Chromatographic techniques have also been used to fractionate or pre-concentrate natural water samples with subsequent application of methods for direct measurement of free metal ion in different fractions (Donat et al., 1997; Gordon et al., 1996 and Gordon et al., 2000).

The first approach has been the most common in investigations of metal speciation in natural systems. The latter has been used to infer the nature of metal-organic complexes based on their physical-chemical behavior during chromatographic separation and/or to isolate complexes of interest for further characterization.

2.1.1 Analytical techniques based on measuring free or labile metal

Techniques based on the response to the *free aquo ion* include ion selective electrodes (ISE) and bioassays. Although ISE is sensitive to very low concentrations of the free metal ion, proper electrode response requires total concentrations greater than those in unpolluted natural waters. This technique has been used for investigation of Cu^{2+} and Pb^{2+} complexation with natural organic matter at elevated metal concentrations (Logan et

al., 1997; Cabaniss and Shuman, 1988; Turner et al., 1986). Comparable results (concentrations of labile Cu) have been obtained for Cu complexation by model ligands and fulvic acids by ISE and differential pulse anodic stripping voltammetry (DPASV), which indicates that the methods are consistent when data sets used for DPASV and Cu ISE measurements overlap (Rozan et al., 1999).

Bioassay studies are based on the toxicity of free metal ions to aquatic microorganisms quantified, for example, by decreased uptake of some substrate for growth. These methods presume that hydrophobic metal complexes capable of diffusion through cell membranes are not present (Byrne, 1996 and ref. cited). In most bioassay studies, strong organic complexing agents are added to some samples so that the biological response to the (known) metal speciation in the amended sample can be compared with the response in the original sample. Results of bioassay studies have been shown to be comparable with those obtained using fixed potential amperometry (Hering et al., 1987).

A relatively new technique for detection of heavy metal ions at low concentrations involves sensors based on proteins with distinct binding sites for metal ions (Bontidean et al., 1998). The proteins are immobilized on a gold electrode which is sensitive to Cu, Cd, Hg, and Zn ions at femtomolar concentrations. The upper concentration limit of the electrode is 10^{-10} M, which presumably corresponds to near saturation of the protein binding sites.

Voltammetric techniques are the most widely used for investigation of metal complexation in natural waters and detect both the free aquo metal ions and *labile metal complexes* (e.g., chlorides, carbonates and hydroxides).

DPASV employs either a hanging mercury drop electrode or a rotating glassy carbon electrode coated with a thin film of mercury. Dissolved metals are electrochemically reduced and amalgamated with the mercury electrode, which essentially preconcentrates the metal and thus increases sensitivity. The oxidative current is measured as the metal is electrochemically stripped from the amalgam. Usually, the increase in peak current is followed as the sample is titrated with added metal. The detection window for this method is determined by the electrode and applied current. DPASV detects metals complexed by both inorganic and organic ligands if the complexes dissociate rapidly in the solution or if the metal is released from the complex directly at the electrode surface. Use of a rotating disk glassy carbon electrode with a deposited thin mercury film can decrease the problem of overestimation of free metal ion concentration. Rapid rotation of the electrode (about 4000 rpm) generates a thin diffusion layer. Since the residence time with the diffusion layer is on the order of 10 ms, metal complexes with dissociation rate constants of less than 1 s^{-1} are not detected (Donat et al., 1995; Rozan et al., 1999).

Cathodic stripping voltammetry (CSV) is a very versatile electrochemical method and has been used to investigate Al, Cd, Cr, Co, Cu, Fe, Ni, Pb, Pt, Sb, Se, Sn, Ti, V, and Zn complexation in seawater (Donat and Bruland, 1995). When the technique is used for investigation of metal interactions with sulfides or thiols, the ligands deposited at the electrode are directly measured after the sample acidification (Luther et al., 1996; Ciglenecki and Cosovic, 1996; Rozan et al., 1999; Rozan and Benoit, 1999; Al-Farawati and van den Berg, 1997, 1999). In all other metal complexation studies, the method is used in conjunction with competitive ligand equilibration (CLE).

In CLE-CSV, a chelating agent or an ion exchanger is added to the sample. The metal present in the sample re-equilibrates between the added and natural ligands. The complex with the added ligand is adsorbed on a hanging mercury drop electrode held at a fixed potential and is quantified by measurement of the peak current during subsequent reductive stripping. The measured signal, corresponding to the complex concentration, is followed as the metal concentration is increased in metal titration experiments. Competing ligands of different strength can be used to examine metal complexation by different fractions of the natural ligand pool. Combined CLE – CSV technique allows investigation of metal speciation at total metal concentrations in the sub-nanomolar range, which is required to probe the strong ligand pool. However, the obtained speciation results can not necessarily be extrapolated to higher total metal concentrations at which complexes with unidentified weak ligands could become important (Miller and Bruland, 1997). Cu speciation measurements in estuarine waters using CLE-CSV (with different competing ligands) and anodic stripping voltammetry (ASV) (with different electrodes) has demonstrated that these techniques give consistent results (Bruland et al., 2000). An important concern with CLE is formation of mixed complexes involving both natural and added ligands. This problem is potentially more significant when added ligands are bidentate.

Although the electrochemical methods are very sensitive, they usually do not allow direct measurements of labile metal species at their ambient total concentrations in unpolluted natural waters. To obtain ambient concentrations of the inorganic metal, the signal (e.g., peak current) is measured as the sample is titrated with added metal. A

model of this response (see section 2.2.4) is used to predict the inorganic (and free) metal concentration at the ambient total metal concentration.

'Pseudopolarograms' (i.e., plots of anodic stripping peak current vs. deposition potential) have been used to determine thermodynamic stability constants from half-wave potentials. The half-wave potentials observed in the natural water sample are compared against those obtained for model ligands with known stability constants. This type of approach has been used in studies on Fe^{3+} catecholate complexes (Taylor et al., 1994), Fe^{3+} hydroxamate complexes (Spasojevi et al., 1999), Zn^{2+} complexes (Lewis et al., 1995), and Cu^{2+} complexes in seawater and algal culture media (Croot et al., 1999). The limitations of using pseudopolarograms include difficulties in distinguishing between kinetic and thermodynamic contributions to complex stability and possible reductive decomposition of the inert complexes, which would lead to the pseudopolarograms of the ligands themselves and not the complexes. Comparison of CLE-CSV measurements with the results of pseudopolarograms indicate that only a single class of ligands is detected by CSV whereas the pseudopolarograms reveal several distinct ligands (Croot et al., 1999). This approach can be complementary to titration approaches based on competitive ligand exchange.

Generally, the electrochemical methods allow quantification of metal interactions with natural ligands but do not provide information on the nature of these complexes. Another important limitation of these methods is that such measurements are usually performed with a single metal and do not consider metal competition.

2.1.2 Analytical approaches based on physical separation

Chromatographic techniques have been used for pre-concentration (i.e., partial isolation) and fractionation of both ligands and metal-organic complexes. Complexed metals have been pre-concentrated by retention on hydrophobic (C₁₈ or XAD-4) and anion-exchange resins (MacKey and Higgins, 1988; Xue and Sigg, 1999; Groschner and Appriou, 1994; Atallah et al., 1991). Metal-chelating resins (Chelex) have been used for pre-concentration of labile metals (Groschner and Appriou, 1994). Immobilized metal affinity chromatography has been employed for isolation of natural ligands (Donat et al., 1997, Gordon and Dyer, 1994; Grodon et al., 1996; Gordon et al., 2000). Metal-binding properties and metal-content of isolated/pre-concentrated samples can be further investigated. Conditional stability constants of isolated samples are usually determined using electrochemical techniques (Xue and Sigg, 1999; Donat et al., 1997, Gordon and Dyer, 1994; Grodon et al., 1996; Gordon et al., 2000).

Chromatographic techniques used for fractionation of metal species are based on size (size-exclusion chromatography, SEC) or polarity (using C₁₈ reverse phase chromatography) (Groschner and Appriou, 1994; Atallah et al., 1991). The distribution of metals among various fractions is determined by quantification of metal content with detection techniques such as atomic adsorption spectroscopy or inductively coupled plasma mass spectrometry for metal determination in different fractions (see Chapter 3, Table 1). Information on the nature and sources of the metal complexes can be inferred from their behavior during sample fractionation. With multi-element detection, all of the metals associated with individual fractions can be measured and their behaviors can be compared.

For example, Rottmann and Heumann (1994) have measured concentrations of various metals in size-fractionated freshwater samples using size-exclusion chromatography directly coupled with inductively coupled plasma mass spectrometry.

Another advantage of chromatographic methods is that they focus on the complexed forms of metals, which are predominant for many metals in natural waters, rather than on minor species such as free or labile metals.

However, dilution of samples during fractionation has limited studies of natural water samples containing low concentrations of metals (Gardner et al., 1982; Zernichow and Lund, 1995; Rottmann and Heumann, 1994).

Another limitation of chromatographic methods is the violation of equilibrium conditions during the separation of metal and ligand species on the chromatographic column. This can lead to partial or complete dissociation of complexes that are labile under the experimental conditions and to alteration of the equilibrium speciation (see Chapters 3, 4, and 5). These artifacts seriously constrain the interpretation of results of chromatographic separation.

The sensitivity of chromatographic separation to the kinetic characteristics of metal complexes can provide some additional information on their dissociation kinetics. The dissociation kinetics of Cu and Pb complexes have been studied using an amberlite XAD-8 microcolumn technique. The technique covers the time scale of measurements between 0.02 and 3 s and allows differentiation of metal complexes into four lability categories: labile, quasi-labile, slowly labile and inert (Procopio et al., 1997).

Chromatographic techniques have been used for the determination of conditional stability constants. Conditional stability constants of copper with naturally-occurring ligands in seawater samples were determined in competitive ligand equilibration experiments using a combination of C₁₈ reverse phase chromatography with atomic adsorption spectrometry (Sunda and Hanson, 1987). SEC has been employed for both determination of molecular size of metal-organic complexes and for measurements of complexation equilibrium constants in biochemical systems (Yoza, 1973 and ref. cited) and for humic and fulvic compounds derived from soils, water and sediments (Buffle, 1988). For the determination of conditional stability constants, the size-exclusion column is pre-equilibrated with an eluant containing a metal buffer. The ligand of interest is injected into the column and reacts with the metal creating a localized change in metal speciation. The metal complex formed by this reaction elutes from the column at its characteristic elution time and, at this time, increases the metal concentration passing the detector over the concentration of the metal in the eluant. The localized region of metal depletion (generated by complex formation) is advected at the flow velocity of the eluant. Measurements of metal concentration eluted from the column allow calculation of a conditional stability constant for the complex. The metal buffer also preserves the dynamic equilibrium between the complex and the metal. This method is, however, problematic for natural water samples containing a mixture of metals at low concentrations.

2.2 Experimental observations

Metal complexation has been investigated with whole water samples, both natural water and culture media (e.g., Croot et al., 1999), and with isolated (i.e., partially purified) components of whole waters (e.g., humic substances isolated on XAD resins or fractionated samples) (Xue and Sigg, 1999; Wells et al., 1998; Gordon et al., 2000). Complexation of various metals, including Cu, Ni, Cd, Pb, Fe, Zn, Hg, Co, Al, and Mn, has been investigated. Varying results have been reported for natural waters with different levels of metal pollution, biological productivity, dissolved organic matter concentrations and for seawater as compared with freshwater.

It has been commonly reported that more than 99% of Cu, 70 – 95% of Pb, 75 – 85% of Cd, 50 – 97% of Zn are present in a non-labile form in seawaters (Bruland et al., 2000; Kozelka and Bruland, 1998; Byrne, 1996; Bruland, 1989; Donat et al., 1995; Donat et al., 1997; Wells et al., 1998). Most of the Cu present in both unpolluted and contaminated freshwaters has been reported to be strongly complexed (Rozan and Benoit, 1999; Xue and Sigg, 1998; Breault et al., 1996).

In contrast, studies in which hydrophobic resins, such as C₁₈-bonded silica or XAD-4, have been used to isolate organically-complexed metals have indicated that the strongly-bound metal fraction constitutes only 10-30% of Cu, 5-10% of Zn, and less than 5% of Fe and Ni (MacKey and Higgins, 1988). This discrepancy could be attributed to the hydrophilic nature of the organic metal complexes or to colloids (Mackey and Zirino, 1994).

Remarkable specificity has been observed in voltammetric studies of metal complexation. Competition between metals (added at environmentally-relevant concentrations) was not observed. For example, Cu could be added to seawater samples without increasing the labile Zn concentration by displacing Zn from organic complexes. Theoretically Cu should form more stable organic complexes than Zn based on the Irving-Williams scale of reactivity (Byrne, 1996).

Another complication in metal speciation studies is variable kinetics of metal coordination. Reported equilibration times of EDTA added to seawater are on the order of 24 hours (Sunda and Huntsman, 1991). Slow equilibration of added metals with ligands present in seawater samples has been demonstrated for Zn, Pb, Ni, and Cu (Muller and Kester, 1991; Capodagilo et al., 1990; Lavigne et al., 1987; Mills et al., 1982). However, there are also observations which indicate that complexation processes in some cases are relatively rapid. Results of Cu(II) titrations of seawater samples from the northeast Pacific were consistent for 5.5 min and 24 hour pre-equilibration times (Coale and Bruland, 1988). Similarly, Zn (Bruland, 1989) and Cd complexation (Bruland, 1992) showed no significant changes in titration characteristics using equilibration periods between 10 min and 24 hours.

The observed strong complexation of metals corresponding to the presence of metals in non-labile forms has been attributed to strong inorganic complexation (specifically by sulfide), formation of colloids, or organic complexation.

2.2.1 Sulfides

Studies of Cu sulfide complexation in four southern New England rivers using CSV have demonstrated that up to 60% of the dissolved Cu is complexed by sulfide (Rozan and Benoit, 1999). It is likely that the sulfide in these oxic waters is derived from biologically-produced sulfur compounds, which are formed by assimilatory sulfate reduction (Dyrssen and Wedborg, 1989). The persistence of sulfides at nanomolar concentrations in oxic waters is a result of slow sulfide oxidation. Cu-sulfide complexes have been shown to be stable in the water column for 21 days (Rozan et al., 1999). The extent of metal-sulfide complexation was reported to be linked to the ambient conditions (including season) and the extent of watershed development. Since no free HS^- was measured in the water, it was suggested Cu-sulfide complexes were not formed in the water but rather released from some source into the water.

Conditional stability constants of metal-sulfide complexes were determined by two different methods using CSV for detection of free sulfide and CLE-CSV measurements with 8-hydroxyquinoline as a competing ligand. These experiments suggested that up to 29% of Cu was bound with sulfide in seawater (Al-Farawati and van den Berg, 1999).

Metal-sulfide complexes were also found to be present in a colloidal fraction (molecular weight > 3 kDa). Although aqueous clusters have been observed during the formation of Zn and Cu sulfides (Luther et al., 1999; 2000), they are not larger than 3000 Da. The presence of metal-sulfide complexes in the colloidal fraction was explained by the formation of an organic layer on the reactive surface of metal-sulfide clusters (Rozan

et al., 1999). Such an organic layer could stabilize the metal-sulfide complexes as compared with those formed in laboratory experiments (Buffle et al, 1998).

2.2.2 Colloids

Formation of colloidal species has been suggested as an explanation of the chemical inertness of metals in natural waters and of the discrepancies between electrochemical and chromatographic investigation of strong metal complexation (Mackey and Zirino, 1994). Traditionally, colloidal fraction has been isolated by filtration through a 0.45 μm filter. Small colloidal particles including some microorganisms and protein fragments pass through such filters. Filtration through 0.2 μm filters and ultrafiltration through membranes with nominal molecular weight cut-offs of 8, 3 and 1 kDa have been used to separate dissolved and colloidal metal species (Wells et al., 2000; Wells et al., 1998; Greenamoyer and Moran, 1997; Rozan and Benoit, 1999).

In regions with high particle concentrations (1 - 10 mg/L) such as near-shore and estuarine waters, it has been shown that between 10 and 30% of Cd, Cu, and Ni may exist in the colloidal size range (1 kDa to 1 μm) (Greenamoyer and Moran, 1997). The colloidal fraction (1 kDa - 0.2 μm) constituted 96% of Fe, 44% of Cu, 25% of Ni, 7% of Zn and 4% of Mn in Narragansett Bay (Wells et al., 2000). Further sample fractionation into large (8 kDa - 0.2 μm) and small (1 - 8 kDa) colloids has demonstrated that metals were not distributed equally between colloidal size classes; colloidal Zn was associated with larger colloids (> 90%), Fe and Ni were found mostly in large colloidal fraction (70 - 85%) but also in the smaller one, while more than 70% of colloidal Cu was associated

with smaller colloids. Another study in Narragansett Bay demonstrated that 50% of the chelated Cu was associated with small colloids (1 -8 kDa) and about 40% of Pb with larger colloids (8 kDa - 0.2 μ m) (Wells et al., 1998).

The three main types of colloids suggested to be present in aquatic systems include compact inorganic colloids (which could provide surface area for formation of organic layers), large, rigid biopolymers (proteins and polysaccharides), and either soil-derived fulvic compounds or autochthonous refractory organic matter (Buffle et al., 1998). Each type of colloid can be stable for weeks to months. Since biopolymers are most likely to be released from plankton under specific conditions such as nutrient deficiencies (Strycek, et al., 1992), it can be expected that aggregation rates will vary seasonally and from one ecosystem to another. As a result the importance of colloids in metal complexation would also vary seasonally and by location.

2.2.3 Organics

Complexation of metals by strong organic ligands is the most common interpretation of experimental observations of metal speciation. The evidence supporting organic complexation includes: partial extractability of trace metals into an organic phase, adsorption onto hydrophobic resins and elution from them by organic solvents, increase of labile fraction of metals after acidification, and chemical oxidation and UV photooxidation, chemical inertness of metal complexes (MacKey and Zirino, 1994). The interpretation of strong metal complexation in terms of formation of organic complexes has been based partly on the similarity of metal titrations of whole water samples and isolated or-

ganic fractions (Cabaniss and Shuman, 1988). However, a recent comparison of isolated humic substances with the ligands present in natural waters conducted at low total metal concentrations indicate that the strongest natural metal-binding ligands are not isolated by standard procedures (Xue and Sigg, 1999).

Although the strong metal complexation observed in natural waters is most commonly attributed to organic complexation the structures of these ligands have not been determined. Such characterization poses significant challenges. The estimated concentrations of organic ligands in seawater are less than 100 nM and less than 10 nM for the strongest ligands (Byrne, 1996). A variety of reactive trace elements (Al, Cr, Mn, Ni, Cu, Zn) have concentrations which are comparable to the total concentrations of this highly reactive class of ligands (Phinney and Bruland, 1994).

2.2.4 Modeling

Metal coordination with natural ligands is likely to involve a wide variety of coordinative site types with a range of affinities for metals. The distribution of these sites is typically deduced from observations of the changing extent of metal complexation as the total metal concentration is increased incrementally. Often such metal titration data can be interpreted with similar success by physically sound models and those that are undoubtedly simplistic. Five different models applicable to analysis of copper complexation by fulvic acid have been compared on statistical grounds by Turner et al. (1986). The models included: a) a multi-site model in which metal and organic matter are assumed to interact at a small number of site-types, each site having an associated stability

constant and total site concentration; b) a multi-dentate model in which the ligand of a single site-type can form either 1:1 or 2:1 complex with the metal; c) an electrostatic model that involves a single binding site with a stability constant that is functionally dependent on the extent of metal occupation of the binding site; d) a normal distribution model which assumes a continuous variation in binding affinities that are normally distributed as a function of log of their stability constant; e) an affinity spectrum model which assumes a continuous variation in binding site affinities with an unknown distribution. Since the necessary structural information on fulvic acid is unavailable, the physical and chemical significance of the models could not be compared. However, based on practical considerations (conceptual and mathematical simplicity) and statistical considerations, the multi-site model was the most satisfactory model for describing the Pb and Cu titration data. Quantitative use of complexation models in natural water systems has been, for the most part, confined to relatively simple multi-site models, which provide parameters (i.e., stability constants and total binding site concentrations) that can be incorporated into equilibrium modeling programs. The number of distinct ligands required to fit titration data depends on the range of total metal concentrations examined rather than on the presence of distinct ligand classes. In general, one ligand is needed for each order of magnitude in the total metal concentration (Dzombak et al., 1986). In most cases, titration data of natural water samples are modeled by assigned stability constants and total concentrations for strong and weak binding sites.

The reported conditional stability constants for the weaker class of ligands range between $10^{8.3} - 10^{10.6}$ and those for the stronger class of ligands are typically on the order of 10^{12} or greater (Byrne, 1996). The parameters extracted by both discrete and continu-

ous ligand models are very sensitive to experimental error (Cernik et al., 1995). It has been demonstrated that, in waters where heterogeneous mixtures of ligands (such as humic substances) are present, distinctions between strong and weak ligand classes are arbitrary and accurate parameters for strong discrete ligands can not be extracted (Voelker and Kogut, 2000).

2.3 Organic ligands in natural waters

The strong metal complexation observed in natural waters has been attributed to various types of organic ligands including biogenic or anthropogenic ligands with well-defined structures and geopolymers of undefined structures. The relative importance of these different types of ligands may vary depending on the ecosystem, the level of anthropogenic inputs, and season.

2.3.1 Well-defined ligands

The synthetic organic ligand that is most widely dispersed in aquatic systems is ethylenediaminetetraacetic acid (EDTA). This compound is used in large quantities and is not removed during wastewater treatment (Breault et al., 1996; Rozan and Benoit, 1999; Bürgisser and Stone, 1997). EDTA has been detected in wastewater effluents at concentrations as high as 19 μM (Kari and Giger, 1996; Nowack et al., 1996). The conditional stability constants of metal-EDTA complexes in natural waters are weaker (by

several orders of magnitude) than those reported for ligands in natural waters (e.g., van den Berg et al., 1990; van den Berg, 1993). This discrepancy might be due, in part, to the slow (re)equilibration of metal species under analytical conditions. For example, in CLE-CSV experiments, the interpretation of analytical data and determination of conditional stability constants assumes that equilibrium is reached between the competing ligand and the ligands present in the sample. However, under the conditions encountered in the CLE-CSV experiments performed with San Francisco Bay water (in which dimethylglyoxime was added as a competing ligand to a pre-formed NiEDTA^{2-} complex), NiEDTA^{2-} appeared to be stronger than predicted by equilibrium calculations due to slow exchange reactions of the complex (Bedsworth and Sedlak, 1999).

This complex can account for strong Ni complexation in San Francisco Bay during the summer when the surface runoff is low and the discharge of wastewater containing elevated concentrations of NiEDTA^{2-} is high (Bedsworth and Sedlak, 1999). Concentrations of EDTA were reported to exceed those of Ni suggesting that this ligand could be important in the complexation of other trace metals.

Studies of Cu speciation in four southern New England rivers have demonstrated that the levels of EDTA were highly dependent on the extent of watershed development (Rozan et al., 1999). Concentrations of EDTA were not detectable (< 1.0 nM) in areas of low development and as high as 60 nM in industrialized areas. Although it was calculated that EDTA could account for up to 75% of Cu complexation in the absence of competing metals, Ni or Fe, even at nM concentrations, could outcompete Cu for EDTA. Surface waters in developed areas tended to have high concentrations of both EDTA and trace metals (including Ni and Fe). Strong synthetic ligands such as EDTA are only

likely to dominate metal speciation in heavily developed areas where surface waters receive significant inputs of sewage effluent.

In unpolluted aquatic systems, such as open ocean waters, the naturally-occurring ligands are thought to have a biological origin (Wells et al., 1998, Gordon et al., 2000; Croot et al., 1999). Since these strong ligands have not been isolated and characterized, their chemical structure(s) and properties are not known. Information is available on the concentrations and conditional stability constants of these ligands, and on their hydrophobicity (Mills et al., 1982; Hanson and Quinn, 1983; Donat et al., 1986) and molecular weights (e.g., Gordon et al., 1996; Wells et al., 1998). The biological origin of these ligands is supported by their specificity for individual metals, their relatively small size (< 1 kDa), the correlation of their maximum concentration with the chlorophyll maximum in oceanic surface waters, and their similarities with the ligands observed in laboratory culture studies.

Biological systems have evolved molecules that form highly stable complexes with a specific metal (Crumbliss, 1991; Silva and Williams, 1991), consistent with the high specificity observed for strong natural ligands. Active transport across cell membranes is constrained in part by molecular size. The limit of about 1 kDa for actively-transported molecules is consistent with the small size of the majority (90%) of strong ligands for Cd and Zn and of 50% of strong Cu-binding ligands reported to be present in seawater (Wells et al., 1998; Wells et al., 2000). The observed maximum concentration of strong ligands occurs near the chlorophyll maximum (Donat and Bruland, 1992; Coale and Bruland, 1988; Moffett et al., 1990), suggesting a recent biological source.

The questions of the biological functions of these ligands and how they get into the water column are still unresolved. Microorganisms in laboratory culture are known to produce specific biomolecules in response to the presence (or absence) of trace metals. These biomolecules may be involved in metal acquisition (e.g., siderophores) (Davis and Byers, 1971; Luckey et al., 1972; Murphy, 1976; Simpson and Neilands, 1976; Trick, 1989; Reid and Butler, 1991), or can alleviate metal stress. The intracellular protein metallothionein and the peptide phytochelatin (Zenk, 1996 and ref. cited; Ahner et al., 1994) and the extracellular Cu-binding protein of *Vibrio alginolyticus* (Harwood-Sears and Gordon, 1990; Harwood and Gordon, 1994) are induced by metal stress.

Laboratory studies indicate that picoplankton (the $< 2 \mu\text{m}$ component of the phototrophic planktonic microflora), specifically cyanobacteria, *Synechococcus* spp., produce Cu ligands similar in strength to those observed in natural waters (Moffett and Brand, 1994, 1996; Moffett et al., 1990; Gordon and Dyer, 1994; Gordon et al., 1996; Croot et al., 1999; Gordon et al., 2000). Heterotrophic marine bacteria and fungi have also been reported to produce dissolved, high affinity copper ligands (Sunda and Gessner, 1989; Gordon et al., 2000).

The strong thiol-containing ligand glutathione is present in living cells at mM concentrations and has been measured at nM concentrations in seawater (Le Gall and van den Berg, 1998). Biosynthesis of the peptide phytochelatin from glutathione is induced by many metals including Cd, Cu, and Zn (Zenk and ref. cited, 1996). Ahner et al. (1994, 1995) have reported that phytochelatin in eukaryotic phytoplankton cultures could be measured even when no metal was added. It has been found in particulates isolated from natural waters with concentrations ranging from 2 to 50 μmol of phytochelatin/g Chl a

(Ahner et al., 1997; Knauer et al., 1998). The highest concentrations were observed where toxic metal concentrations were elevated (Ahner et al., 1994). Measured concentrations of phytochelatin have shown a systematic variation with free cupric ion concentrations in New England harbors (Ahner et al., 1997). These works clearly indicate that phytochelatin is produced under natural conditions at levels that are related to free Cu^{2+} concentrations in the external environment. Since phytochelatin is produced inside cells under reducing conditions, their presence outside the cells in oxic waters is questionable. Export of Cd-phytochelatin from the marine diatom *Thalassiosira weissflogii* has been demonstrated but only at inorganic Cd concentrations several orders of magnitude higher than those observed in seawater (Lee et al., 1996). Similar metal-phytochelatin transport across phytoplankton membranes could occur for Zn or Cu complexes. Such release, at ambient metal concentrations, could occur as a result of cell breakage by zooplankton grazing or viral lysing. It is not known, however, whether such complexes would persist in oxic ocean waters or whether their conditional stability constants are within the range reported for the extracellular ligands that are thought to control metal speciation in natural waters.

2.3.2 Ill-defined ligands

Although highly metal-specific strong ligands play a significant role in metal complexation, there also appears to be a contribution from relatively non-specific ligands with a wide spectrum of site characteristics. This class of ligands is more abundant but has weaker conditional binding constants ($10^{8.3} - 10^{10.6}$) than the strong specific ligands

(Byrne, 1996). For highly reactive trace metals, such as Cu, this class of ligands becomes important when the stronger ligands are saturated.

Terrestrial humic substances have been proposed as the weaker class of ligands in natural waters (e.g., Sedlak et al., 1997; Rozan and Benoit, 1999; Moffet et al., 1997). Humic substances derive from the decomposition of plant and animal matter. Their size, molecular weight, elemental composition, structure, and number and position of functional groups vary depending on the origin and age of the material. Humic materials consist of a skeleton of alkyl/aromatic units cross-linked mainly by oxygen and nitrogen groups with the major functional groups being carboxylic acid, phenolic and alcoholic hydroxyls, ketone, and quinone groups (Gaffney et al., 1996).

Humic substances account for a significant portion of total dissolved organic carbon (DOC) in rivers and estuaries - between 30 and 60% depending on salinity (Moran et al., 1991). Typical DOC concentrations in rivers and estuaries are on the order of 2 to 20 mg/L. Recent studies of Cu complexation by humic substances in freshwater and seawater have demonstrated the presence of strong binding sites that may be important in Cu binding at total humic concentrations as low as 1 mg/L (Xue and Sigg, 1999; Kogut and Voelker and Kogut, 2000).

Whether the humic substances play an important role in metal complexation depends on their concentration, the presence of other strong ligands, and the concentration of trace metals. Such ill-defined ligands are likely to be important for aquatic systems with elevated metal and DOC concentrations.

2.4 Concluding remarks

Investigations of metal speciation in different aquatic systems have suggested that metal speciation can be controlled by well-defined (synthetic and biogenic) organic ligands, ill-defined organic ligands (i.e., humic substances) and inorganic ligands (e.g., sulfides). However, the relative contribution of the possible ligands to overall metal complexation will depend on the type of natural water system and specifically on the extent of anthropogenic impact, biological activity, and DOC content. It may be expected that synthetic organic ligands, such as EDTA, will be important in highly developed areas with significant inputs of sewage effluent while biogenic organic ligands can control metal speciation in both unpolluted and impacted natural waters. Colloids are most likely to be important in waters with high rates of particle formation such as estuaries. Ill-defined organic ligands such as humic substances can also significantly influence metal speciation especially in natural waters with high DOC content and elevated metal concentrations.

Chapter 3

DIRECT QUANTIFICATION OF METAL-ORGANIC INTERACTIONS BY SEC AND ICP-MS

(This chapter includes the paper written with Janet Hering which is to appear in "Journal of Environmental Quality")

3.1 Introduction

The speciation of metals (i.e., their distribution among various organic and inorganic forms) profoundly influences the mobility of metals in natural waters and their bioavailability. Many laboratory studies have demonstrated that metal-organic complexes (which are commonly hydrophilic) are not available for uptake by biota, hence metal toxicity is proportional to the free metal ion concentration or activity. This mode of metal toxicity is supported by field studies of Cd bioavailability in freshwater (Hare and Tessier, 1996) and of the production of phytochelatin (which is taken as a proxy for trace metal uptake) in New England harbors (Ahner et al., 1997). It has been suggested that many metals (particularly copper, nickel, cadmium and zinc) are strongly complexed by organic ligands. The concentrations of strong organic ligands in natural waters have been estimated to be comparable to the ambient concentrations of trace metals. Due to the numerous analytical problems and the complexity of natural systems, measurements of metal speciation in natural waters are extremely difficult and the understanding of metal interactions on the molecular level remains limited.

The application of chromatographic methods to study the distribution of metals among fractions of organic matter has been reviewed by Mackey and Higgins (1988). Size-exclusion chromatography (SEC) has been applied to determine the molecular size/weight of natural organic matter (NOM). Although this technique is subject to artifacts caused by solute-gel interactions (Miles and Brezonik, 1983; Peuravuori and Pihlaja, 1996), molecular weights can be determined for NOM by SEC that are consistent with those measured by vapor pressure osmometry, low angle X-ray scattering, ultracentrifugation and field flow fractionation (Chin et al., 1994; Pelekani et al., 1999).

Although some sample dilution is unavoidable during chromatography, direct coupling of a chromatography column with a metal-detection technique minimizes such dilution; this approach has been applied to both natural and biological samples (Table 3.1). These methods provide information on the distribution of metal(s) between different organic fractions. For geochemical modeling of equilibrium metal speciation, however, information is needed on the complexation capacity and metal affinity of naturally-occurring ligands.

SEC has been used to determine conditional stability constants of both humic and fulvic acids and well-defined organic ligands (Mantoura and Riley, 1975; Adamic and Bartak, 1984). In these experiments, metal buffers were added to preserve the dynamic equilibrium between the complex and the metal but this method is problematic for natural water samples containing a mixture of metals at low concentrations. Without metal buffers, equilibrium conditions are necessarily violated during chromatographic separation of metal species. If, however, the dissociation kinetics of natural metal complexes are sufficiently slow that the complexes are stable within the time frame of the experiments, con-

ditional stability constants can still be determined. Other applications of chromatography to determine conditional stability constants of naturally-occurring ligands without metal buffers have been based on competitive ligand equilibration (Sunda and Hanson, 1987; Sunda and Huntsman, 1991).

This paper describes the development of an SEC/ICP-MS method for the determination of the conditional stability constants of metal complexes with strong organic ligands. The method is validated with well-defined organic ligands in ligand-competition studies with a single metal and a binary metal mixture. Experiments with ligands forming less stable and/or more labile complexes illustrate the limitations on applicability of this method and on previous interpretations of similar chromatographic techniques.

Table 3.1. Determination of metal speciation with combined chromatographic - metal detection techniques

Sample	Analyte	Chromatography	Detector	Ref. [†]
Proteins (albumin, ferritin) and human plasma amended with metals	Zn, Cu and Cd	SEC	FAAS	a
Freshwater	Cu, Ni, Mo, Pb, Cr, Fe	SEC	ICP-ID-MS	b
Freshwater, seawater	Cu, Cd, Pb and Mn.	C ₁₈ , anion-exchange, Che-lamine	ETAAS	c -
EDTA and ammonium pyrrolodine-dithiocarbamate amended with metals	Cu and Pb	Silica, C ₁₈	ETAAS	d
Pig kidney extracts	Cd	SEC	ICP-MS	f
Freshwater	Al	SEC	GFAAS, UV [‡]	g
Pond water	Mo, Ba, Sr, Cu, Ca, Si, Mn, Al, Zn, Fe	SEC	ICP-MS	h
Pond water	Fe, Al, Mn, Zn, Ni, Cu, Sn, Pb, Mo, V, Ti, Ce, Co, Cd, Y, La, U	SEC	ICP-MS	i
Seawater	Cu, Zn, Fe, Ni, Mg	C ₁₈ , XAD-2, SEC	FAFS [§]	j
Metal-phytochelatin complexes	Cd, Zn, Cu and Pb	SEC	AAS	k
Seawater	Cu	C ₁₈	GFAAS [§]	l
Tap water	Cr(VI)	C ₁₈	DLAAS	m

[†] (a) Ebdon et al., 1987; (b) Rottmann and Heumann, 1994; (c) Groschner and Appriou, 1994; (d) Atallah et al., 1991; (f) Crews et al., 1989; (g) Zernichow and Lund, 1995; (h) Itoh et al., 1993; (i) Itoh et al., 1996; (j) Mackey and Higgins, 1988; (k) Scarano and Morelli, 1997; (l) Sunda and Hanson, 1988; (m) Zubin et al., 1998.

[‡] post-column reaction with pyrocatechol violet for Al UV detection;

[§] chromatographic column not directly coupled to metal detection.

Abbreviations:

FAAS – flame atomic absorption spectrometry; ETAAS – electrothermal atomic absorption spectrometry; AAS – atomic absorption spectrometry; GFAAS – graphite furnace atomic absorption spectrometry; FAFS – flame atomic fluorescence spectrometry; ICP-(ID)-MS – inductively coupled plasma (isotope dilution) mass spectrometry; SEC – size – exclusion chromatography; DLAAS – diode laser atomic absorption spectrometry; C₁₈ – hydrophobic resin.

3.2 Materials and methods

3.2.1 Reagents and standard solutions

The chromatographic elution solutions (10 mM ammonium acetate) were prepared by titrating acetic acid (99.7 vol. %; Seastar) with ammonium hydroxide (21.7 vol. %; Seastar) to $\text{pH} = 6.0$ and diluting with ultrapure water (18 M Ω , Millipore Milli-Q system). Stock solutions and standards were prepared gravimetrically in the elution solution from 1 g L⁻¹ Cu or Ni reference solutions (Fisher Scientific), ethylenediaminetetraacetic acid (EDTA, 98%, Sigma), ethylenediiminobis (2-hydroxyphenyl) acetic acid (EDDHA, 97%, Aldrich), 8-hydroxyquinoline-5-sulfonate (HQS, 98%, Aldrich), or nitrilotriacetic acid (NTA, A.C.S. reagent, Baker Analyzed). The standard tuning solution for ICP-MS (500 ppb of Li, Y, Tl) was prepared from 1000 mg L⁻¹ solutions of these elements (ULTRA Scientific). The internal standard solution was prepared from a 10,000 mg L⁻¹ Ga standard solution (ULTRA Scientific) by dilution in 25% (vol. %) nitric acid (Seastar, Canada). Size calibration of the column was performed using 1.0 g L⁻¹ solutions of polystyrene sulfonate standards (Scientific Polymer Product) and hydrophobic interactions were examined using 1.0 g L⁻¹ solutions of gallic acid (Sigma), phthalic acid, anisic acid, 3-methylsalicylic acid, and 3-phenyl-1-propanol (all from Aldrich, 98% purity).

3.2.2 Instrumentation

A “Protein Purification” FPLC* (Pharmacia Biotech) consisting of an HPLC pump (P-500), a sample injection valve (MV-7) fitted with a 50 μ L loop made of PEEK, and a UV monitor (UV-M II with a 6 μ L cell) was used with a glass TSK 3000 PW size-exclusion column (TosoHaas). The column is packed with hydrophilic, rigid, spherical methacrylate beads with the size of 10 μ M. The column can be operated at 0.4 - 0.8 mL/min flow rate. The system was controlled by an FPLC Controller LCC-501 Plus and the data captured using FPLC Assistant Version 1.00 (Pharmacia Biotech) software. A FRAC-200 fraction collector (Pharmacia Biotech) was used in preliminary experiments. All components contacting the eluent are made of inert materials such as PEEK or PTFE to avoid metal contamination. A low-resolution quadrupole ICP-MS (HP 4500, Hewlett Packard) was used to determine the time-resolved signals at mass-to-charge ratios of 65 for Cu, 60 for Ni and 69 for Ga. Metal concentrations in the eluent were determined by integrating ICP-MS peak areas. The ICP-MS sensitivity was optimized using the standard tuning solution. The operating parameters for the quadrupole ICP-MS and the FPLC system are summarized in Table 3.2.

* FPLC - fast protein liquid chromatography, the name Pharmacia Biotech uses for a size-exclusion chromatography system.

Table 3.2. HPLC and ICP-MS instrumental and operational parameters	
ICP-MS (HP4500)	
rf power	1260-1275 W
nebulizer	cross-flow
spray chamber	CF type (Hewlett Packard)
carrier gas (argon) flow rates	1.16-1.27 L/min
sample cone	nickel
skimmer cone	nickel
measurement parameters	
integration per point	0.1 s
dwel time	100 ms
sweeps per replicate	1
HPLC	
mobile phase	10 mM ammonium acetate
flow rate	0.4 mL/min
wavelength of UV absorption	280 nm
sample loop	50 μ L

3.2.3 Calibration and column characterization

The column void volume (6.9 mL) was established using Blue Dextran 2000 and the total permeation volume (20.5 mL) with acetone. The linear size-exclusion calibration range of the column was determined with polystyrene sulfonate standards (1,640 to 127,000 Da) with detection by UV absorbance at 280 nm. Based on these experiments and on considerations of ICP-MS performance (for which sensitivity and stability are improved at lower salt concentrations), an eluent composition of 10 mM ammonium acetate (pH = 6) was selected. In experiments with HQS, 10 mM sodium acetate in 0.1 M NaCl was used as an elution solution and eluent was collected with a fraction collector rather than being directly introduced into the ICP-MS. Characterization of hydrophobic interactions on the column was performed with small (136 - 170 Da) analytes of varying hydrophobicity.

3.2.4 Chromatographic conditions and coupling to ICP-MS

Before each run the column was cleaned with a 10 mM solution of NTA to avoid metal accumulation, 4 mM sodium azide (Sigma) to prevent microbial activity, and Milli-Q water and was pre-equilibrated with the elution solution. EDTA (5×10^{-5} M) was injected between samples to confirm the absence of metal accumulation on the column. All the experiments with on-line metal detection were performed with 10 mM ammonium acetate at pH = 6 as the elution solution.

For on-line metal detection, the FPLC eluent was delivered to the cross-flow nebulizer of the ICP-MS. Column eluent was acidified in-line, before entering the nebulizer, by the addition of 25% nitric acid (final concentration 1%) delivered into a Y-connection by a peristaltic pump through fine-bore tubing. An internal standard of $500 \mu\text{g L}^{-1}$ Ga was also introduced through this line.

ICP-MS response was calibrated using metals in the presence of a two-fold excess of EDTA. Calibration standards were run through the full chromatographic system to account for the dilution and dispersion of the peaks during chromatography. The validity of this procedure was established in preliminary experiments that demonstrated insignificant changes in Cu recovery for standards with different Cu:EDTA ratios (up to a tenfold excess of EDTA). Cu recoveries of $> 97\%$ were achieved even with only a 5% excess of the ligand. Injections of EDTA on the column after the samples had eluted did not show any Cu in the EDTA peaks, indicating that there was no column contamination from sample dissociation.

3.2.5 Equilibrium speciation calculations

Conditional stability constants were calculated from thermodynamic constants (Martell et al., 1993) and were corrected for ionic strength using the Davies equation. Equilibrium speciation calculations were performed using MINEQL⁺ (Schecher and McAvoy, 1998). For the conditional stability constants (listed in Table 3.3)

$$K^{\text{cond}} = \frac{[\Sigma\text{ML}]}{[\text{M}^{2+}][\Sigma\text{H}_x\text{L}]}$$

the term $[\Sigma H_x L] = [L] + [HL] + [H_2L] + \dots$ incorporates the acid – base speciation of the ligand and $[\Sigma ML] = [ML^+] + [MHL^{2+}] + [MOHL] + \dots$ includes protonated and hydroxylated forms of the metal complex. At pH 6.0, both ligands (EDTA and EDDHA) are present as protonated species, more than 99% of metal–EDTA complexes are present as ML and more than 98% of metal–EDDHA complexes are present as protonated complexes.

Table 3.3. Conditional stability constants of metal complexes studied	
Complex (ML)	Log K_{ML}^{cond}
CuEDTA	15.5
CuEDDHA	14.1
CuNTA	9.6
NiEDTA	14.4
NiEDDHA	8.7
ZnEDDHA	5.8
Cu(HQS) ₂	21.9 [†]

Thermodynamic constants from Martell et al. (1993) were corrected using the Davies equation for ionic strength. Conditional constants calculated for pH = 6.

† Log β_{ML2}

3.3 Results and discussion

The SEC/ICP-MS method developed for characterization of metal-organic complexes relies on the competition between organic ligands for one or more metals. A requirement of this method is that the complexes are sufficiently kinetically inert to remain intact during SEC despite the perturbation of equilibrium conditions that necessarily occurs during the chromatographic separation. The method was validated and its limits established with well-defined organic ligands forming 1:1 or 1:2 metal-ligand complexes.

3.3.1 Ligand competition with a single metal

Complexes of Cu with the organic ligands EDTA and EDDHA were separated by SEC and the metal in each complex quantified by ICP-MS. The peaks corresponding to the complexes were well resolved with the CuEDTA complex eluting at an elution volume (V_{EL}) of 7.0 mL ahead of the CuEDDHA complex ($V_{EL} = 9.3$ mL).

A series of experiments were performed with varying concentrations of total Cu (in the range of 0.20 to 1.0 μ M) and ligands (in the range of 1.0 to 5.0 μ M). In all cases, the total concentration of ligand that exceeded that of Cu and Cu recovery was > 97%. Although, in this validation study, the conditional stability constants for Cu complexes with both of the ligands were known, EDTA was chosen as the reference ligand Y (Y = EDTA) and experimental values of the conditional stability constant for the Cu complex with the target ligand L, K_{ML}^{cond} , (L = EDDHA) were determined from

$$K_{ML}^{\text{cond}} = \frac{[ML]K_{MY}^{\text{cond}}([Y]_T - [MY])}{([L]_T - [ML])[MY]}$$

where the concentrations of the Cu ($M = \text{Cu}$) complexes with the reference and target ligands, $[MY]$ and $[ML]$, were measured and the total ligand concentrations, $[L]_T$ and $[Y]_T$, and conditional stability constant for the Cu complex with the reference ligand, K_{MY}^{cond} , were known. In 15 separate experiments, the average (geometric mean) value of $\log K_{ML}^{\text{cond}}$ ($M = \text{Cu}$, $L = \text{EDDHA}$) was 14.1 ± 0.2 ; the reported value is 14.1 (Taliaferro et al., 1984). Alternatively, good agreement between observed and predicted Cu speciation in this system can be illustrated by comparing the measured values of $[\text{CuEDTA}]$ with those predicted from the reported value for $\log K_{ML}^{\text{cond}}$ (Fig. 3.1a).

These experiments demonstrate that strong 1:1 complexes are kinetically inert under these chromatographic conditions. For such complexes, conditional stability constants can be determined by ligand competition studies with separation and quantification of metal complexes by SEC/ICP-MS. The organic ligands chosen for this study have conditional stability constants for Cu(II) in the range reported for strong Cu-binding ligands in natural waters (ca. 10^{13} to 10^{15}), thus we anticipate that Cu complexes with naturally-occurring ligands would also be compatible with the SEC/ICP-MS method.

3.3.2 Ligand competition with a binary metal mixture

Although most investigations of metal speciation in natural waters have focused on a single metal, remarkable metal selectivity has been reported in a few studies (Bruland, 1989; Capodaglio et al., 1990; Wells et al., 1998). For well-defined ligands, equilibrium speciation calculations may be used to predict the extent of competition between metals. For naturally-occurring ligands, however, it is not clear that observations of metal complexation in systems with a single dominant metal can be easily extrapolated to systems with competing metals. Thus, it would be advantageous to determine directly the distribution of (competing) metals in various organic complexes.

The applicability of the SEC/ICP-MS method for this purpose was examined in two-ligand, two-metal systems with approximately $1\ \mu\text{M}$ $[\text{Ni}]_{\text{T}}$ and $[\text{Cu}]_{\text{T}}$, $3\ \mu\text{M}$ $[\text{EDDHA}]_{\text{T}}$, and $[\text{EDTA}]_{\text{T}}$ varying from 0.3 to $7\ \mu\text{M}$ (the exact values of concentrations varied but were known to within 1%). The observed concentrations of NiEDTA and CuEDTA complexes were in good agreement with those predicted from equilibrium speciation calculations (Fig. 3.1b). Although the NiEDDHA complex could not be quantified because of its lability on the column (see below), this did not interfere with the quantification of EDTA complexes which elute before the EDDHA complexes. Nevertheless, these experiments demonstrate the general applicability of the method to multi-metal systems.

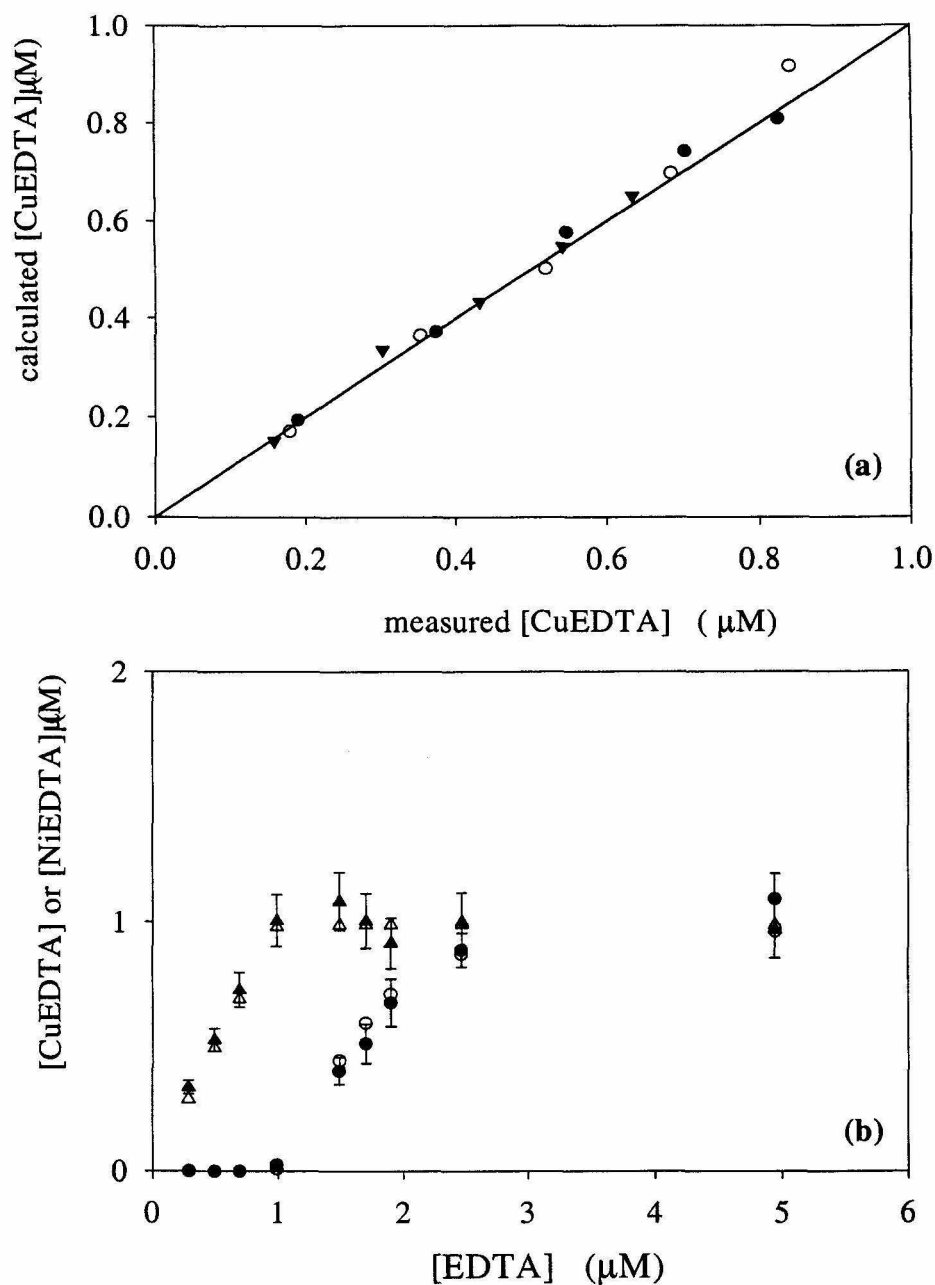


Fig. 3.1

Comparison of measured and calculated concentrations of metal-EDTA complexes. Conditions: pH = 6, I = 10 mM, (a) solutions of EDTA and EDDHA titrated with Cu, $[\text{Cu}]_{\text{T}} = 0.20$ to $1.0 \mu\text{M}$. Symbols: (\bullet) $[\text{EDDHA}]_{\text{T}} = [\text{EDTA}]_{\text{T}} = 1.0 \mu\text{M}$; (\circ) $[\text{EDTA}]_{\text{T}} = 2.0 \mu\text{M}$, $[\text{EDDHA}]_{\text{T}} = 5.0 \mu\text{M}$; (\blacktriangledown) $[\text{EDTA}]_{\text{T}} = 1.0 \mu\text{M}$, $[\text{EDDHA}]_{\text{T}} = 5.0 \mu\text{M}$; (—) 1:1 line; (b) solutions of Cu, Ni and EDDHA titrated with EDTA, $[\text{EDDHA}]_{\text{T}} \cong 3 \mu\text{M}$, $[\text{Ni}]_{\text{T}} \cong [\text{Cu}]_{\text{T}} \cong 1 \mu\text{M}$ (exact values varied but were known to within 1%). Symbols: (\circ) calculated and (\bullet) measured $[\text{CuEDTA}]$, (\triangle) calculated and (\blacktriangle) measured $[\text{NiEDTA}]$.

These studies were carried out with micromolar metal concentrations for method validation and did not focus on maximizing sensitivity. Previous work using a coupled SEC/ICP-MS system has demonstrated that total metal concentrations in fractionated natural water samples could be accurately determined at the level of 10^{-8} M by employing isotope dilution techniques (Rottmann and Heumann, 1994). Determination of conditional stability constants at ambient concentrations of metals and strong ligands in unpolluted natural waters may require some type of sample preconcentration.

3.3.3 Method limitations due to complex lability during SEC

Equilibrium conditions are necessarily violated during chromatographic separation of complexes on the column. Although Cu complexes of strong ligands such as EDTA and EDDHA are sufficiently kinetically inert that the species distribution of the original solution is maintained during chromatography, weaker metal complexes may undergo dissociation. In experiments with the moderately strong ligand NTA, recovery of Cu was very poor (5 – 10%), indicating substantial dissociation of the complex during the chromatographic separation. Injections of 5×10^{-5} M EDTA after the CuNTA samples resulted in the elution of Cu peaks which decreased with successive injections. This recovery of Cu confirms the dissociation of the CuNTA on the column. In experiments with an even weaker complex, ZnEDDHA, no signal from the complex could be detected indicating complete complex dissociation.

A less pronounced effect of complex lability was observed for NiEDDHA in the 2-metal, 2-ligand experiment described previously. In this experiment, three Ni peaks

were observed in the ICP-MS chromatogram (Fig. 3.2), with the peak eluting earliest corresponding to NiEDTA. The second, small peak can be attributed to the labile NiEDDHA complex which undergoes partial dissociation on the column. This peak coelutes with the CuEDDHA complex but contains only 3-5% of the Ni expected to be present as the EDDHA complex on the basis of equilibrium calculations. The third peak is attributed to Ni released from the EDDHA complex and eluted as inorganic species and/or acetate complexes (derived from the elution buffer). The observed lability of the NiEDDHA complex(es) may be related to the weak conditional stability constant ($K_{ML}^{cond} = 10^{8.7}$) and to protonation of the complex. Total Ni recoveries, for a range of experimental conditions, were between 85 and 102%. In the absence of any (strong) organic ligand, a single peak for Ni was observed in the ICP-MS chromatogram with an elution volume of 16.2 mL, similar to the elution volume of the third peak (15.9 mL) in the experiments with EDDHA.

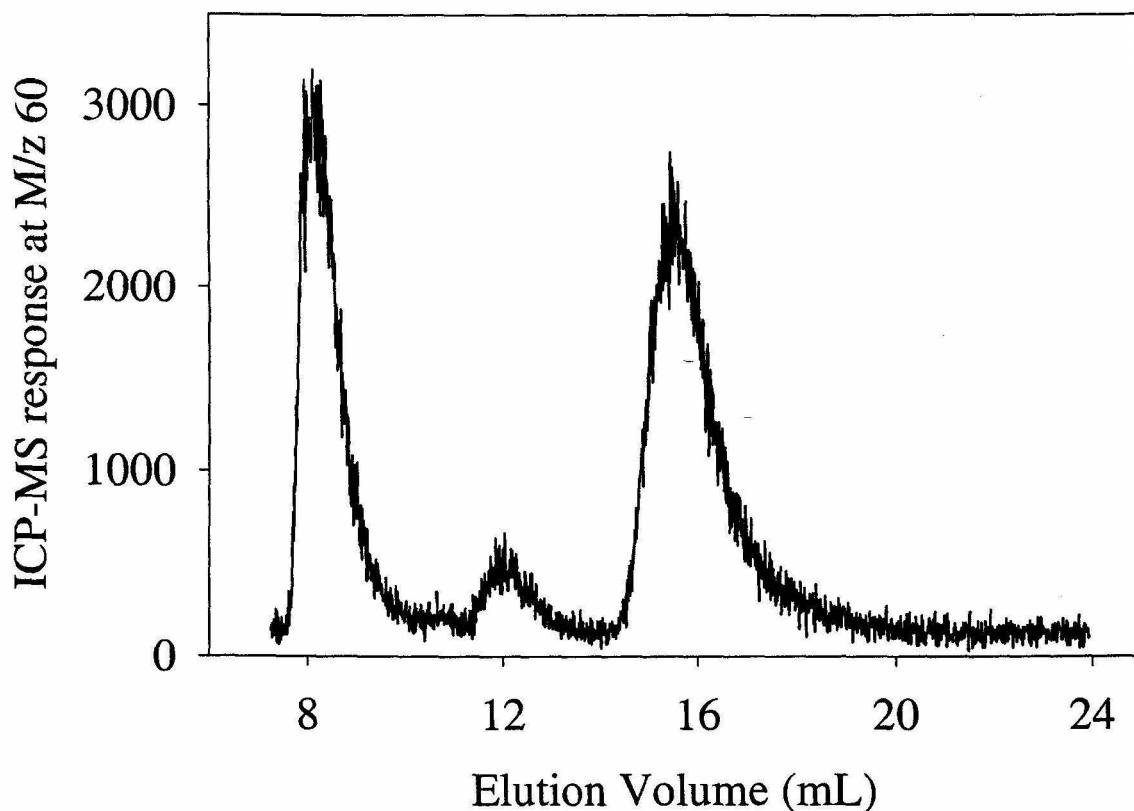


Fig. 3.2 ICP-MS chromatogram obtained for Ni – EDTA – EDDHA system. Conditions: $[\text{Ni}]_{\text{T}} = 0.990 \mu\text{M}$, $[\text{Cu}]_{\text{T}} = 0.990 \mu\text{M}$, $[\text{EDTA}]_{\text{T}} = 0.30 \mu\text{M}$, $[\text{EDDHA}]_{\text{T}} = 3.0 \mu\text{M}$, $\text{pH} = 6$, $I = 10 \text{ mM}$.

For most 1:1 complexes the free (or protonated) ligand and the metal-ligand complex are not sufficiently different in size to be separated by size exclusion on the column. In these cases, disequilibrium arises from separation of the free metal from the (co-eluting) free and complexed ligands. However, it may also be the case that the free and complexed ligands are separated on the column. Such behavior was observed with the 1:2 complexes of Cu with HQS because of the size difference between $\text{Cu}(\text{HQS})_2$ and the free ligand. Despite the fairly high formation constant for the 1:2 complex (Table 3.3), partial dissociation of the complex on the column was observed. A preliminary

study was conducted with total Cu and HQS concentrations sufficient to allow both UV and ICP-MS detection of complexes. The eluent was collected with a fraction collector and the concentration of Cu in each fraction was determined by ICP-MS. The UV chromatogram (Fig. 3.3) shows two irregular peaks.

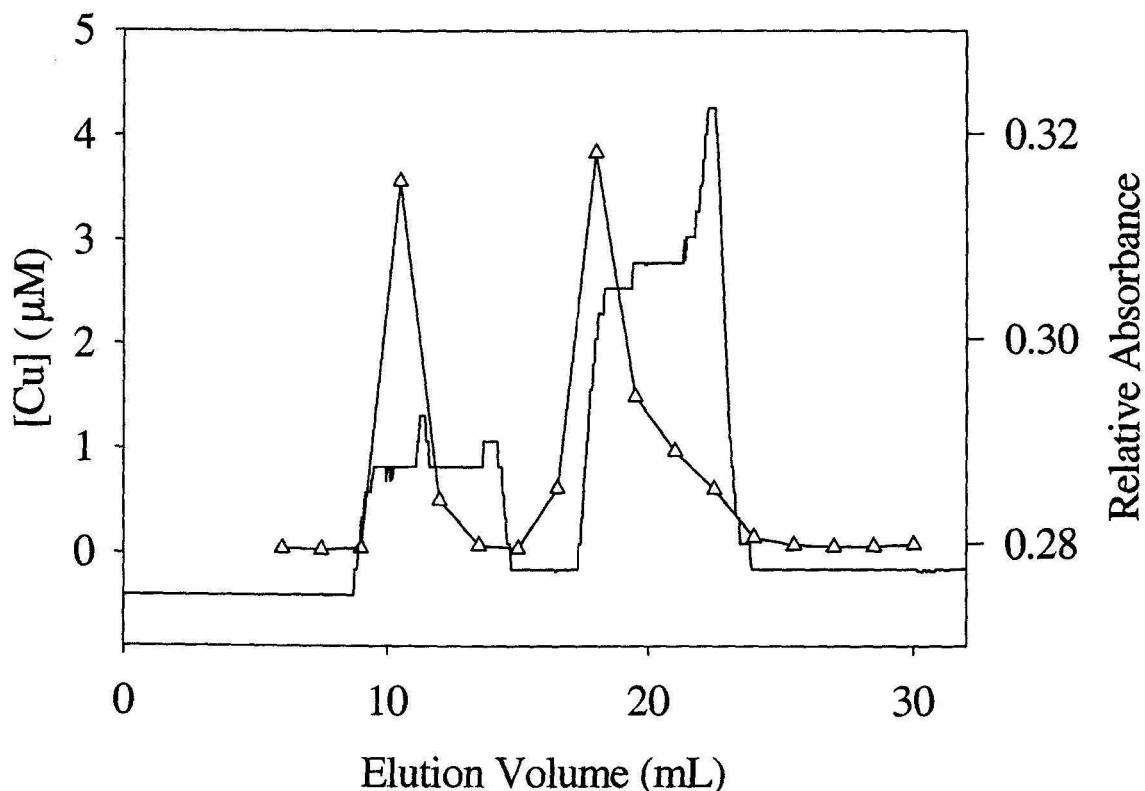


Fig. 3.3 UV chromatogram obtained with SEC separation of $\text{Cu}(\text{HQS})_x$ and HQS and ICP-MS measurements of Cu in different fractions. Conditions: $[\text{Cu}]_{\text{T}} = 0.40 \text{ mM}$, $[\text{HQS}]_{\text{T}} = 1.0 \text{ mM}$, $\text{pH} = 6$, $I = 0.10 \text{ M}$ (NaCl). Symbols: (Δ) $[\text{Cu}]$, measured with ICP-MS after fraction collection; (—) UV absorbance of HQS and $\text{Cu}(\text{HQS})_x$ in column eluent.

The first peak corresponds to the original $\text{Cu}(\text{HQS})_2$ complex which, as the largest molecule, elutes first. The second peak can be attributed to the free ligand and to a secondary complex formed on the column as the free ligand reacts with the Cu released

by dissociation of the initial 1:2 complex. This secondary complex gives rise to the shoulder on the leading edge of the second peak detected by UV and to the second Cu peak detected with ICP-MS. Although these experiments show that the coupled SEC/ICP-MS method is subject to significant kinetic limitations, the kinetic window of the method should be appropriate for the strong ligands postulated to control metal speciation in natural waters.

3.3.4 Method limitations due to incomplete separation of complexes by SEC

Resolution of metal complexes with the reference ligand and the ligand of interest on the column is a necessary condition for the determination of conditional stability constants and for the study of metal-organic interactions by this method. The model molecules examined in this study, EDTA and EDDHA, are very similar in their molecular weight (336 Da and 360 Da respectively) and, on the basis of the molecular weight calibration of the column, would be expected to have the same elution volume of 7.9 mL. Nevertheless, both the free ligands and their Cu complexes show well resolved peaks on the chromatograms, with EDTA eluting before the calculated elution volume, at 7.0 mL, and EDDHA significantly later, at 9.3 mL.

The chromatographic behavior of analytes can be influenced by their hydrophobicity and charge due to hydrophobic and charge-exclusion interactions with the column resin. For low molecular weight analytes with the octanol-water partitioning

coefficients varying by several orders of magnitude, the observed elution volumes varied by a factor of three (Fig. 3.4). Thus, separation of various complexes does not always result solely from size exclusion. At pH 6.0, EDTA and its metal complexes are negatively charged, which results in charge exclusion and early elution, while EDDHA and its metal complexes are largely uncharged and have aromatic character, which leads to hydrophobic interactions with the column media and retarded elution.

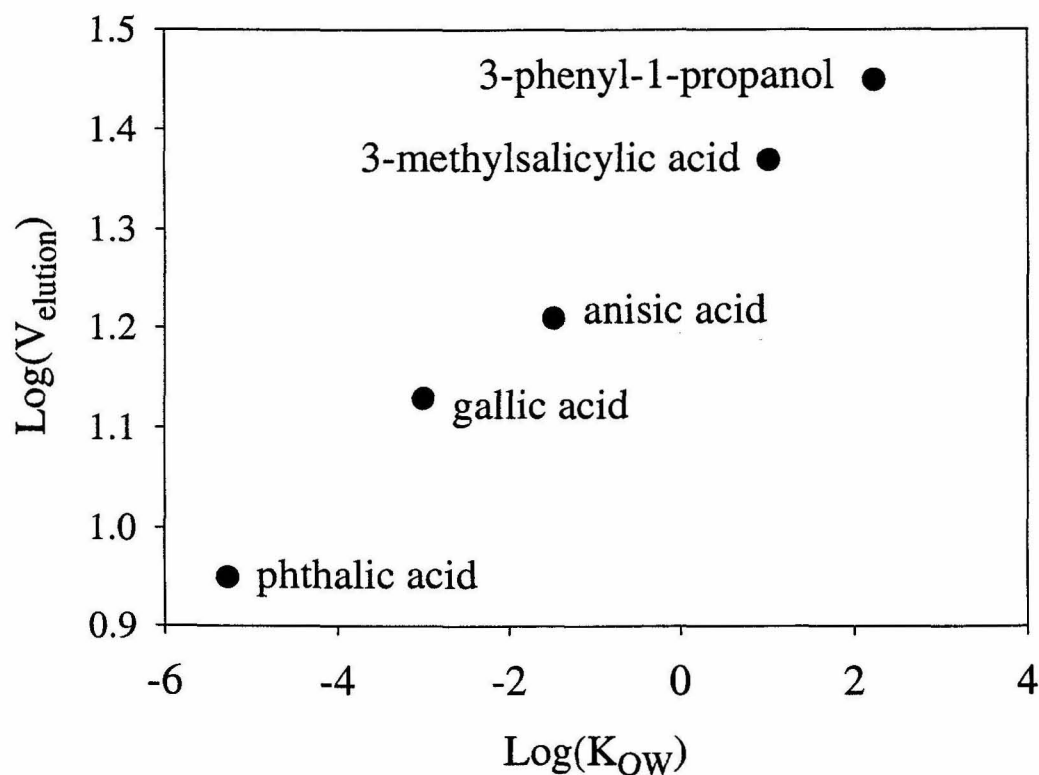


Fig. 3.4 Elution volumes for various compounds of comparable molecular weight as a function of their octanol-water coefficients illustrating hydrophobic interactions on TSK 3000 PW (pH = 6, I = 10 mM).

Indeed, on the basis of the octanol-water partitioning coefficient for EDDHA ($\log K_{ow} = -1.84$, estimated from structure-property relationships) and observed correlation of elution volumes with $\log K_{ow}$ (Fig. 3.4), the expected elution volume for EDDHA would

be even greater than that observed. However, since the large, highly aromatic molecule vitamin B12 did not exhibit any retardation due to hydrophobic interactions (data not shown), it appears that such interactions are strongly size dependent. Thus it is reasonable that EDDHA, with a molecular weight of 360 Da, would be less subject to hydrophobic effects than the lower molecular weight compounds (136 – 170 Da) used to examine hydrophobic interactions with the column.

These observations with well-defined metal-organic complexes illustrate the range of chromatographic behavior that may be expected for metal complexes with dissolved organic matter (DOM). Although the mechanism for separation of metal-ligand complexes is not crucial for the SEC/ICP-MS method, correlation between elution times/volumes and molecular weight should be viewed with caution, particularly for compounds with longer retention times/volumes.

3.3.5 Implications of experimental results for previous studies

The results presented here highlight the need to account for the kinetic lability of target metal-organic complexes and suggest some important limitations on previous interpretations of the chromatographic behavior of metals associated with natural organic matter.

Chromatographic separation has previously been coupled with a metal detection technique to determine metal speciation (Table 3.1). The present work, however, demonstrates that non-equilibrium conditions during chromatographic separation can result in partial or complete loss of a metal from labile complexes and redistribution of

the metal between different chromatographic fractions. Complex dissociation during chromatographic separation would be evidenced by poor mass balance, such as partial metal recovery in an individual chromatographic run and recovery of excess metal in the subsequent runs due to column contamination. In a study of Cd speciation in pig kidney extract, replacement of the guard column and extensive column cleaning was required after each run to obtain reproducible UV and ICP-MS chromatograms with Cd recoveries of 75-100% (Crews et al., 1989). Similarly only partial Pb recovery was observed during fractionation of freshwater samples on a size-exclusion column and Pb was detected in subsequent EDTA injections (Rottmann and Heumann, 1994).

For labile metal-organic complexes, dissociation of the complex and interaction of the metal with the column may result in complete loss of the metal to the column or elution of the metal at late elution volumes/times. In studies of metal complexation by phytochelatin, formation of metal complexes was demonstrated by polarography; the affinity for the metals decreased for $\text{Cu} > \text{Pb} > \text{Cd} > \text{Zn}$ (Scarano and Morelli, 1997). In the same study, only the Zn complex was not recovered in size-exclusion chromatography, suggesting dissociation of this complex. The chromatographic behavior of metals during fractionation of seawater samples on a size-exclusion column was found to vary from complete loss to elution at volumes exceeding the total permeation volume of the resin depending on the eluent composition (Mackey and Higgins, 1988).

Loss of metals from labile complexes to chromatographic columns may be detected by mass balance calculations and injections of strong complexing ligands on the column after the separation. However, redistribution of a metal between different chromatographic fractions may be more difficult to assess. Several peaks of Cu and Zn

(Itoh et al., 1996), of Cu, Mo and Ni (Rottmann and Heumann, 1994), and of Mn, Zn, Cu, alkali and rare-earth elements (Itoh et al., 1993) were observed in water samples fractionated on size-exclusion columns. Although these peaks were attributed to different DOM constituents, it is also possible that the partial dissociation of early-eluting metal complexes resulted in co-elution of the released metals with DOM eluting later. For DOM fractions with unknown metal affinity, it is difficult to draw conclusions about distribution of specific metal binding sites, especially for metals, such as Zn, which generally form weaker organic complexes. With independent data on the metal affinities of different chromatographic DOM fractions, more accurate interpretation of experimental results should be possible.

3.4 Conclusions

A coupled SEC/ICP-MS method allows the determination of the distribution of metals among competing strong ligands where metal complexes are separated by SEC and the metal associated with each complex is quantified by ICP-MS. With an appropriate reference ligand, the method can be used to determine the conditional constant(s) of a target ligand(s). The method is, however, subject to kinetic limitations, and can only be applied to strong organic complexes which are inert under chromatographic conditions. The possibility of dissociation of metal complexes during chromatographic separation and redistribution of metals, particularly from early-eluting to late-eluting ligand fractions, must be considered when the chromatographic behavior of metal-organic complexes is interpreted. The insights gained in this study of well-

defined organic ligands provide the basis for extending this method to naturally-occurring organic ligands.

Acknowledgements

This project is funded “in part” by a grant from the National Sea Grant College Program, National Oceanic and Atmospheric Administration, U.S. Department of Commerce, under grant number NA66RG0477, project number R/CZ – 146 through the California Sea Grant College System. The views expressed herein are those of the authors and do not necessarily reflect the views of NOAA or any of its sub-agencies. The U.S. Government is authorized to reproduce and distribute for governmental purposes.

APPENDIX 3A

CALIBRATION OF TSK GEL 3000 PW COLUMN WITH POLYSTYRENE SULFONATE STANDARDS

This appendix contains calibration curves of TSK GEL 3000 PW size-exclusion column obtained with different compositions of the elution solution (water, 10 mM ammonium acetate and 10 mM sodium acetate in 0.1 M NaCl). Based on these experiments composition of the elution solution for the initial experiments with HQS was chosen to be 10 mM sodium acetate in 0.1 M NaCl. All the experiments using a coupled SEC/ICP-MS technique, with eluent being directly introduced into the ICP-MS, were performed with 10 mM ammonium acetate based on consideration of improved sensitivity and stability of ICP-MS at lower salt concentrations.

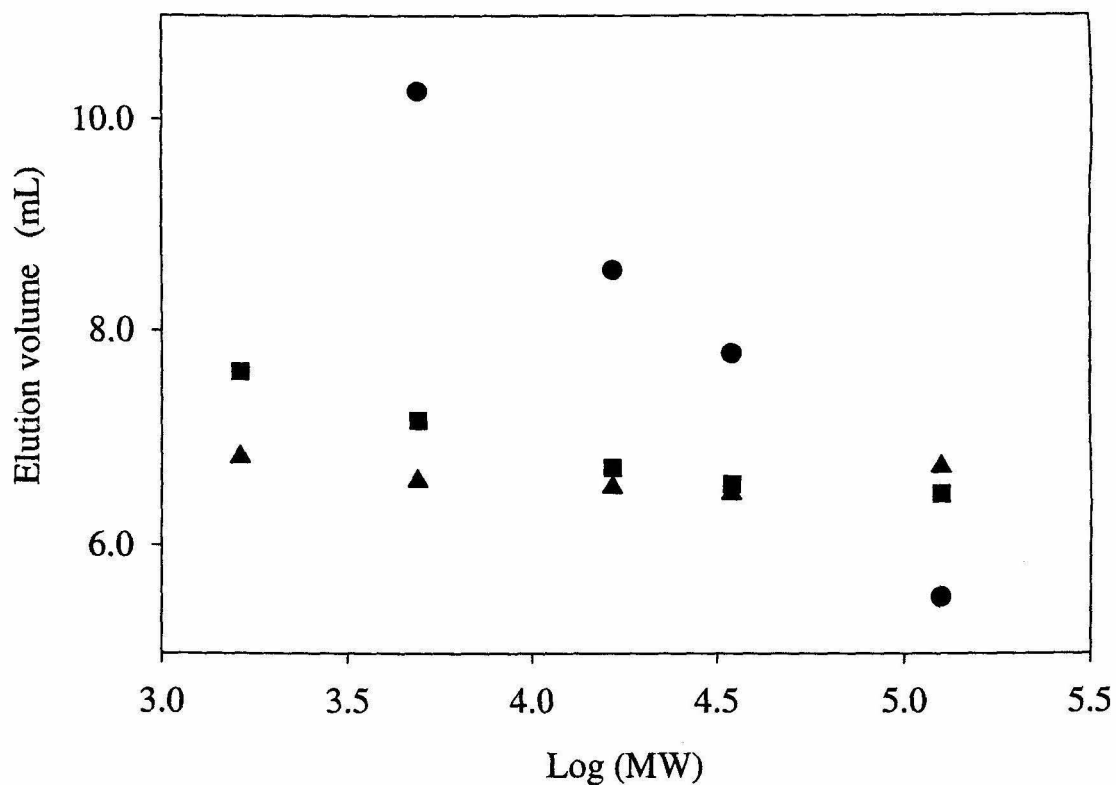


Figure 3A.1. Calibration curves of TSK GEL 3000 PW size-exclusion column with polystyrene sulfonate standards in water (▲), 10 mM ammonium acetate (■), and 10 mM sodium acetate in 0.1 M NaCl (●).

APPENDIX 3B

SEPARATION OF ANALYTES WITH SIZE-EXCLUSION CHROMATOGRAPHY (SEC)

A simplified scheme of the size-exclusion chromatography system used in the study is shown in Fig. 3B.1. The system consisted of an HPLC pump, a sample injection valve fitted with a 50 μ L loop and a UV detector and was used with a glass TSK 3000 PW size-exclusion column. The column is packed with hydrophilic, rigid, spherical methacrylate gel beads with a 10 μ m diameter. The packing of the column bears a slight negative charge of 5 - 18 μ eq/mL (see also sections 3.2.2 - 3.2.3).

A buffer solution of 10 mM ammonium acetate (pH 6) was pumped through the column at a constant flow rate of 0.4 mL/min for half an hour before sample injection for column pre-equilibration. A sample was introduced into the 50 μ L sample loop with a syringe and then it was automatically injected into the flow of the buffer by the motor valve. For the buffer flow rate used, the injection time was on the order of 8 sec. After separation of analytes, the eluent was delivered to the UV detector and then to the cross-flow nebulizer of the ICP-MS (see also section 3.2.4).

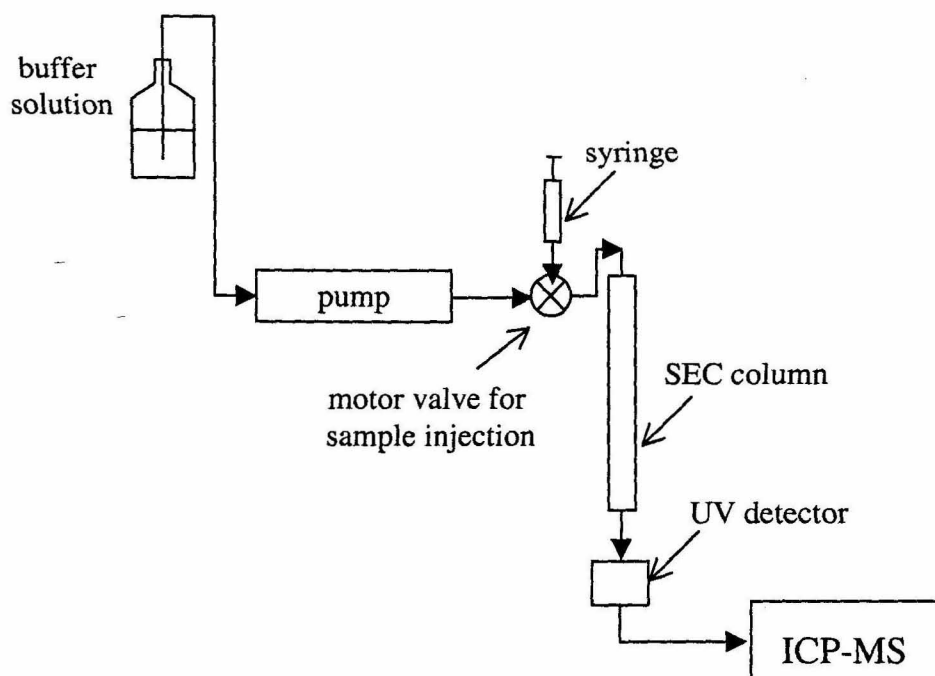


Figure 3B.1. Schematic set-up of the size-exclusion chromatography system used in the study.

In an idealized case, the transport of analyte molecules through the column may be described by conventional passage along the interstices surrounding the gel particles and delays caused by the diffusion into size-discriminating particle pores (Fig. 3B.2). For large molecules, which are incapable of entering the pores, the elution volume is defined as the void volume of the column (determined in this study with Blue Dextran 2000, see section 3.2.3). Small molecules, which have free access to all pores, define the permeation volume of the column (determined in this study with acetone, see section 3.2.3).

Molecules of intermediate size, which have partial access to the pores, elute at some time between that for large and small molecules (le Maire et al., 1995).

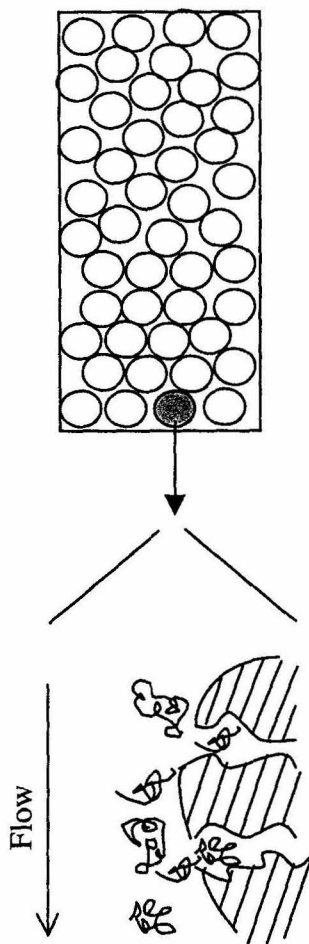


Figure 3B.2. Scheme for the separation of molecules on a size-exclusion column.

Since the stationary phase of the size-exclusion column used in this study has some negative charge and consists of organic gel, analytes also experience ionic and hydrophobic interactions with the particles of the stationary phase (Potschka and Dubin, 1995 and ref. cited). In the case of hydrophobic interactions, the analyte molecules are adsorbed on the surface of the gel particles, which causes their elution at later times than

expected based on their size. In the case of ionic interactions, positively-charged analytes are retarded on the column while the negatively-charged analytes experience repulsion from the stationary phase and are excluded. Such interactions lead to the elution of charged analytes at later times (for positively-charged analytes) or earlier times (for negatively-charged analytes) than expected based on their size (see also section 3.3.4).

Chapter 4

MATHEMATICAL MODELING OF THE CHROMATOGRAPHIC BEHAVIOR OF METAL COMPLEXES

4.1 Introduction

Metal-organic complexes are thought to dominate metal speciation (i.e., the distribution of metals among various organic and inorganic forms) for a variety of trace metals (Wells et al., 1998; Kozelka et al., 1997; Byrne, 1996 and ref. cited; Bruland, 1992; Capodaglio et al., 1990; Bruland, 1989; Coale and Bruland, 1988). This contention is supported by various types of evidence, such as the partial extractability of trace metals into an organic phase, adsorption of metals onto hydrophobic resins and elution from them by organic solvents, the increase of labile fraction of metals after acidification, chemical oxidation and UV photooxidation, and chemical inertness of metal complexes (MacKey and Zirino, 1994 and ref. cited).

Metal-organic complexes have been shown to be unavailable for uptake by biota except when the metal-organic complex is a neutral hydrophobic complex that can passively diffuse through cell membranes (Florence et al., 1992; Phinney and Bruland, 1994). In many laboratory studies, it has been demonstrated that metal toxicity is proportional to the free metal ion concentration or activity. This mode of metal toxicity is

supported by field studies of Cd bioavailability in freshwater (Hare and Tessier, 1996) and of the production of phytochelatin (which is taken as a proxy for trace metal uptake) in New England harbors (Ahner et al., 1997). Although organic complexation can greatly decrease a metal's bioavailability and toxicity, the nature of the strong metal-binding ligands is not well understood. Studies of metal complexation have generally characterized such ligands in terms of conditional stability constants and total concentrations determined from metal titration data. Less commonly, metal-organic complexes have been fractionated based on some physical-chemical characteristics.

Chromatographic methods have been applied for the isolation and pre-concentration of metal-organic complexes. Size-exclusion chromatography has been used to determine molecular size and weight of natural organic matter (Chin et al., 1994; Pelikani et al., 1999) and conditional stability constants of both humic and fulvic acids and well-defined organic ligands (Mantoura and Riley, 1975; Adamic and Bartak, 1984). The problems arising from loss or alteration of analyte species during chromatographic separation due to non-equilibrium conditions and solute-gel interactions have been recognized (Mackey and Higgins, 1988 and ref. cited; Miles and Brezonik, 1983; Peuravuori and Pihlaja, 1997).

To minimize sample dilution, methods that couple chromatography with metal detection have been increasingly used for studies of metal complexation in both natural water and biological samples (see Chapter 3, Table 3.1). With these techniques, species present in a sample under equilibrium conditions are separated on a chromatographic column and metal concentrations are directly measured in different fractions. For kinetically-inert complexes, such determinations can accurately reflect metal speciation in the

original solution. For labile complexes, partial or complete dissociation under non-equilibrium conditions on the chromatographic column leads to a discrepancy between the initial equilibrium and experimentally-determined distribution of chemical species. This discrepancy becomes especially important when quantitative results are sought but, in some cases, even qualitative information on metal distribution between different chromatographic fractions may be questionable.

In general, the main concerns in chromatography have been the separation of chemical species and determination of their physical-chemical characteristics with applications limited to analytes that are stable on the column (Fischer 1980, Yau et al., 1979). Thus most of the theoretical analyses of chromatography have focused on a mathematical description of processes leading to zone spreading and tailing effects. The main objectives of such analyses have been to optimize conditions based on resolution and rapid separation or to identify an optimum parameter (size, in the case of size-exclusion chromatography) governing the position of a solute eluting from a chromatographic column (Potschka and Dubin, 1995; Provder, 1987). Only relatively recent advances in instrumental analysis have allowed direct coupling of separation and metal detection techniques for quantitative studies of metal complexation at low metal concentrations. Although the processes leading to the alteration of partially labile analytes during chromatographic separation have been discussed previously (Mackey and Higgins, 1988 and ref. cited), mathematical modeling of these processes in application to coupled separation-metal detection systems has received little attention.

The present work attempts to provide a resolution of the problems that arise in the course of extension of chromatographic methods to analytes which are partially labile on

the column. To account for the differences between the equilibrium and experimentally-determined speciation, a mathematical model of the dynamic processes taking place in the chromatographic column is developed. This discussion focuses on a coupled size-exclusion chromatography-inductively-coupled plasma mass spectrometry (SEC/ICP-MS) system but also applies more generally to other coupled separation-metal detection techniques.

The coupled SEC/ICP-MS system is described in section 4.2. The mathematical description of the system under investigation is found in section 4.3. There, the general principles of diffusion and kinetics (Giddings, 1965) are applied to the SEC/ICP-MS system in order to obtain a system of coupled partial differential equations (section 4.3.1) that describes the evolution of the spatial distributions of the concentrations of the "free" and complexed metal. [Note that the "free" metal is taken to include all forms of metal other than the complex(es) initially present in the sample injected onto the column.] The equation for the concentration of the complex can be solved separately (section 4.3.2), considerably simplifying the subsequent analysis. From this solution, the distribution of the concentration of the "free" metal is obtained via Green's function methods (section 4.3.3). The full solution to the system of equations is rather cumbersome, although it can be simplified in the various regimes of interest. The discussion focuses on the total amount of metal eluted from the column, either in the form of the initial or as "free" metal. The various regimes for which the chromatographic behavior of metals can be analyzed are presented in section 4.3.4, which concludes the purely theoretical part. The experimental data appear in section 4.4 where they are used as input for the equations from the section 4.3 to derive the dissociation rate constant and equilibrium concentration

of the metal complex. The results are summarized and discussed in the concluding section 4.5.

4.2 System description and model assumptions

This section describes the principle of the coupled SEC/ICP-MS technique and introduces the model for this system based on some simplifying assumptions. The model will be expressed in terms of mathematical equations in the next section.

In a coupled SEC/ICP-MS system, chemical species are separated primarily on the basis of size and the associated metals are quantified by integrating peak areas on an ICP-MS chromatogram. In this technique, both complexed and "free" metals are measured by ICP-MS in the form of a metal peak. As an example, Figure 4.1 illustrates that peaks for both complexed and "free" Ni are observed in the chromatogram obtained for a sample containing Ni and two strong organic ligands.

4.2.1 Model parameters

A pre-equilibrated sample containing metal-organic complexes of interest is injected on a size-exclusion column. The model will describe the metal M present as the complex with a concentration $ML(x,t)$ or as the "free" metal with a concentration $M(x,t)$. Here, x denotes the coordinate along the column, $x=0$ corresponds to the injection point and $x=L$ (where L is the length of the column) is the position of the detector. The dead

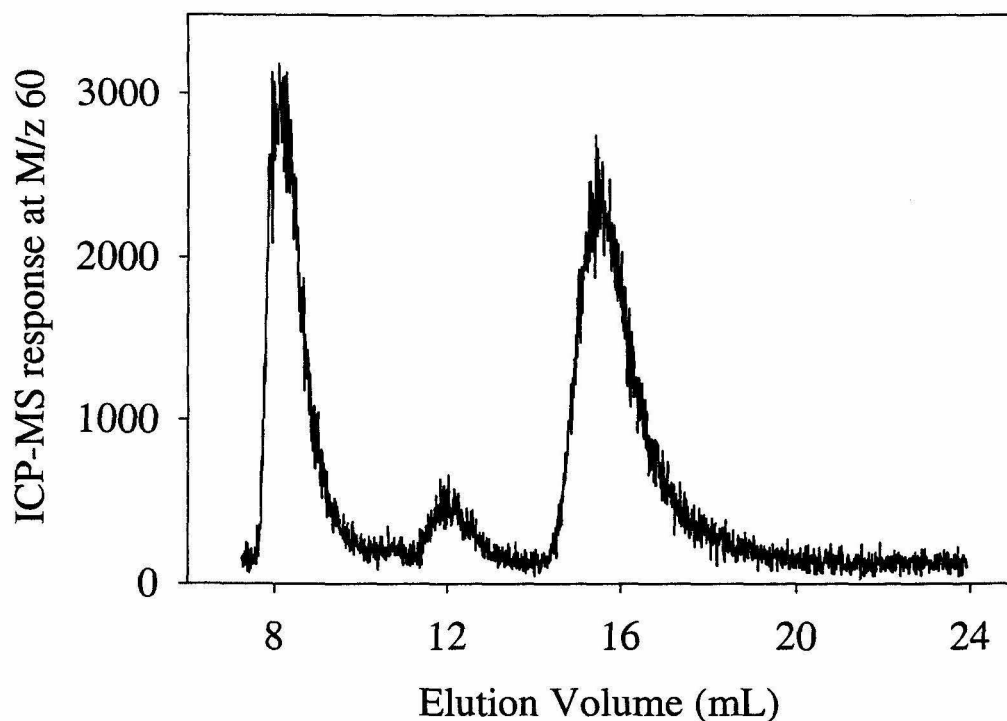


Figure 4.1. ICP-MS chromatogram obtained for Ni-EDTA-EDDHA system. Conditions: $[\text{Ni}]_{\text{T}} = 0.99 \mu\text{M}$, $[\text{EDTA}]_{\text{T}} = 0.30 \mu\text{M}$, $[\text{EDDHA}]_{\text{T}} = 3.0 \mu\text{M}$, $\text{pH} = 6$, $I = 10 \text{ mM}$ (ammonium acetate). The earliest eluting peak corresponds to NiEDTA , the second, small peak to the labile NiEDDHA complex, the last peak corresponds to "free" Ni released from the EDDHA complex and eluted as inorganic species and/or acetate complexes (derived from elution buffer).
 EDTA - ethylenediaminetetraacetic acid;
 EDDHA - ethylenediiminobis (2-hydroxyphenyl) acetic acid.

volume between the column and the detector is negligible. Here, the "concentration" refers to the linear concentration, the amount of metal per unit length in [moles/cm] (Table 4.1). To obtain the usual concentration, in [moles/cm³], the linear concentration is divided by the cross-section of the column. The parameter t denotes time [sec] of travel from the injector ($x = 0$) to the position x .

During chromatography, the following processes govern metal distribution between different fractions (as measured by ICP-MS): solute transport along the column due to eluant flow, complex dissociation due to perturbation of the initial equilibrium conditions which leads to redistribution of the metal between different chromatographic fractions, and metal-column interactions which result in loss of the metal to the column (Fig. 4.2). Other processes that influence the dispersion of the chromatographic peak but do not lead to metal loss or redistribution are of less interest. For that reason, after the general equations describing the system are formulated (in the section 4.3.1), diffusion is neglected (section 4.3.4) to obtain simplified relations that would account for the total metal flux measured by ICP-MS and quantified by the peak area integration.

Analytes are assumed to be moving along the column with some constant effective velocities (v_{ML} for the metal complex and v_M for "free" metal released from the complex, where $v_M < v_{ML}$), which are proportional to the flow velocity. Diffusion coefficients of the metal complex, s_{ML} , and "free" metal, s_M , are assumed to be constant and molecular processes that determine the magnitude of diffusivity are not considered.

The "free" metal can be lost from the mobile phase due to adsorption, which is taken to be irreversible on the time scale of chromatographic separation.

Table 4.1. Symbols used in the text

$\delta(x)$		Dirac's delta function
Δ_M, Δ_{ML}		defined on p.18 of the text
a, b, c		fitting parameters (see page 20)
Erf		error function
$F_{ML}(x, t)$	[moles/sec]	flux of complexed metal through the detector
$F_T(x, t)$	[moles/sec]	flux of total metal through the detector
$G_M(x-x', t-t')$		Green's function
k_b	[sec ⁻¹]	rate constant for dissociation of the metal complex
k_m	[sec ⁻¹]	rate constant for adsorption of the "free" metal onto the column
L		ligand
L	[cm]	column length (distance from the injection point to the detector)
M		metal
$M(x, t)$	[moles/cm]	"free" metal concentration
ML		metal-ligand complex
$ML(x, t)$	[moles/cm]	concentration of metal complex
N	[moles]	equilibrium amount of metal complex in the pre-equilibrated solution injected onto the column
s_M	[cm/(sec) ^{1/2}]	diffusion coefficient of the "free" metal
s_{ML}	[cm/(sec) ^{1/2}]	diffusion coefficient of the metal complex
t	[sec]	time from the sample injection
t'	[sec]	integration variable (time of metal release from metal-ligand complex)
t_M	[sec]	elution time of the peak for the "free" metal in advection-dominated regime
t_{max}	[sec]	elution time of the peak for the metal complex in general case (either advection- or diffusion- dominated)
t'_{max}	[sec]	saddle point of integral determining the metal flux (see Appendix 4A)
t_{ML}	[sec]	elution time of the peak for the metal complex in advection-dominated regime
v_M	[cm/sec]	effective velocity of the "free" metal released from the complex
v_{ML}	[cm/sec]	effective velocity of the metal complex
x	[cm]	position along the column
x'	[cm]	integration variable (position along the column of metal release from metal-ligand complex)

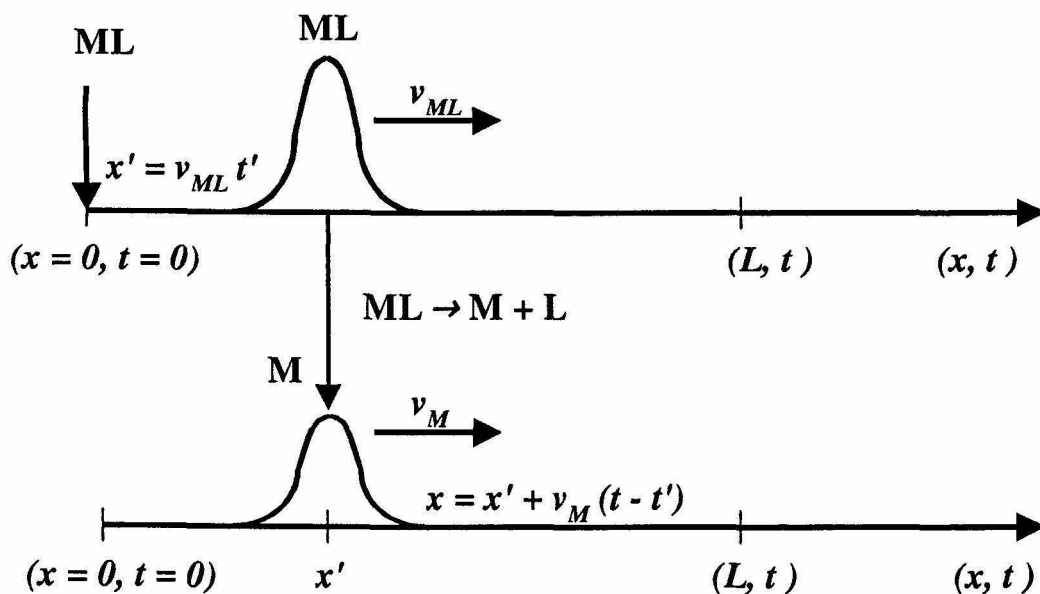


Figure 4.2. Qualitative description of the system. A metal-organic complex (ML) is injected on the column at time $t = 0$ and position $x = 0$. The complex moves with an effective velocity v_{ML} . At time t' and position x' the complex partly dissociates into the ligand (L) and "free" metal (M). Released "free" metal moves with the effective velocity v_M . In the end of the column, (L, t) , both complexed and "free" metal are detected by ICP-MS.

The adsorption rate is assumed to be first order in the "free" metal concentration and is characterized by the adsorption rate constant k_m . This rate constant incorporates both adsorption at the immobile phase surface and retention within the stagnant volumes of pores (which is generally dependent on the flow rate). Within the narrow range of experimental flow rates, however, the adsorption rate constant can be assumed to be independent of flow rate.

This discussion addresses metal-ligand complexes of 1:1 stoichiometry. For such

complexes, the size and thus effective velocity of the complex and the free ligand are similar and they are not separated from each other on the column. The effective velocity of the "free" metal should be significantly smaller resulting in separation of the "free" metal from the complex and/or free ligand on the column.

As the "free" metal is separated from the complex, the initial equilibrium is necessarily perturbed. The consequent dissociation of the complex ($ML \rightarrow M + L$) is assumed to proceed with the first order kinetics with the rate constant k_b . Complex (re)formation is neglected, as the rate of this reaction would be proportional to the concentrations of the "free" metal and free ligand. As the metal is further separated on the column, the product of these concentrations is necessarily small.

The metal-organic complexes of interest are relatively strong, and they taken to be present in the solution with the ligand in excess. Thus metal speciation in the pre-equilibrated solution is dominated by its complexes, so that the initial concentration of the "free" metal is negligible ($M(x,0) = 0$).

With these assumptions, the experimental conditions may be stated in the following way. The complex ML at an initial amount N [mole] is injected at time $t = 0$ at the coordinate $x = 0$ into a mobile phase moving with some constant velocity. Due to the differences in effective velocities, the complex and the ligand are separated from the metal; as a result the equilibrium conditions are perturbed and the complex dissociates with a dissociation rate constant k_b releasing "free" metal and ligand. The "free" metal may be lost to some extent to the column by adsorption. Both the "free" metal and complexed metal are detected by ICP-MS after they are eluted from the column, at $x = L$.

4.2.2 Limiting behavior of metal complexes

There are several limiting cases for the chromatographic behavior of metal complexes. There are two essential regimes of interest, distinguished by the time scale of diffusion relative to that of complex dissociation, the diffusion-dominated and advection-dominated regimes (Fig. 4.3). In the diffusion-dominated regime, advection is slow compared to the effects of diffusion and dissociation; the complex is transported mainly via diffusion, while dissociating. The diffusion-dominated regime is not operative for any of the experiments in the present study.

The advection-dominated regime corresponds to a negligible effect of dissociation on the location of the peak for the complex in the elution profile. In this case, diffusion changes only the shape of the profile. A significant amount of metal may be lost from the complex on the experimental time scale due to the dissociation, but the elution times/volumes of peak maxima are determined by the effective velocities of the analytes.

For the advection-dominated regime a further classification is introduced based on the extent of complex dissociation on the experimental time scale.

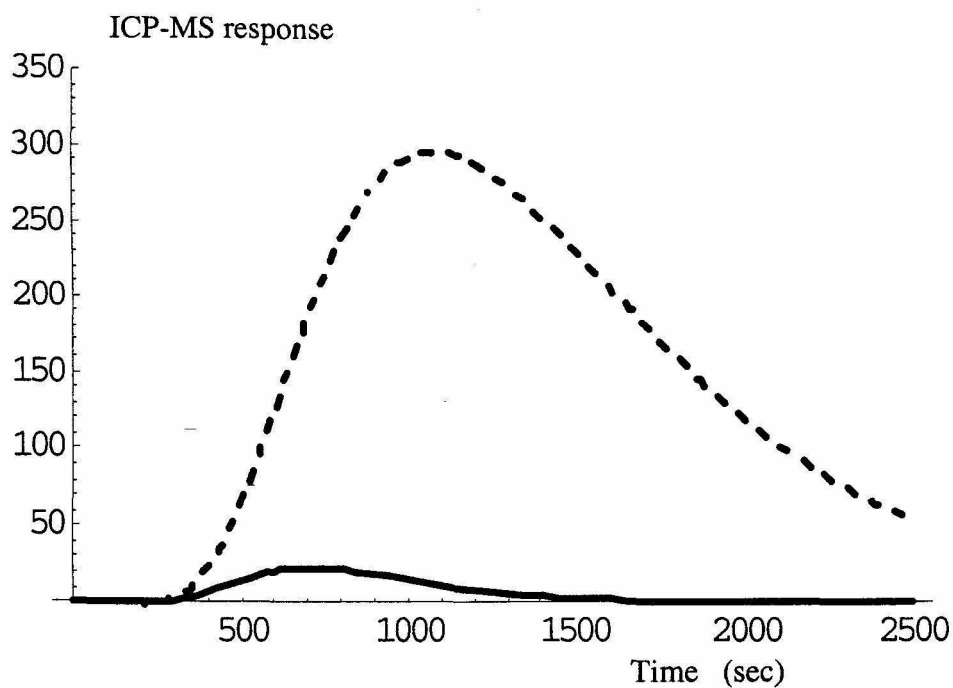


Figure 4.3. Illustration of advection- (---) and diffusion- (—) dominated regimes of metal complex dissociation (Eq. (5)). Note that only the signal corresponding to the metal complex is shown in the graph.
 Values of parameters: $s_{ML} = 0.4 \text{ cm}/(\text{sec})^{1/2}$, $v_{ML} = 0.025 \text{ cm}/\text{sec}$, $k_b = 0 \text{ cm}^{-1}$ (---), $k_b = 0.003 \text{ cm}^{-1}$ (—).
 Note that for diffusion-dominated dissociation the peak of the metal complex elutes before the elution time (t_{ML}) corresponding to the effective velocity of the complex.

The amount of the complex that dissociates during the chromatographic separation is determined by the rate of dissociation of the complex and its retention time on the column which in turn depends on its effective velocity. If only a small amount of the complex dissociates on the experimental time scale, then the complex is called "quasi-inert" (Figs. 4.4 a, b). When most of the complex dissociates during the chromatographic separation, the complex is called "labile" (Fig. 4.4 c). [Note that the details of Figure 4.4 are discussed in section 4.3.4].

For quasi-inert complexes, the shape of the experimental ICP-MS chromatogram is governed by the diffusion coefficient of the complex, with the peak maximum located at the elution time of the complex as determined by its effective velocity. In this case, a constant tail of the "free" metal extending from the elution time of the complex to that of the "free" metal (as determined by the effective velocity of the "free" metal) would be observed in the absence of metal adsorption (Fig 4.4a). Adsorption to the column immobile phase depresses the "free" metal tail with the metal released earlier on the column being lost to a greater extent; thus the observed metal concentration decreases as the elution time of the metal is approached (Fig. 4.4b). [Note that the curves for the "free" and total metal in the figure are obtained with the use of Eq. (24) given in Appendix 4A].

For labile complexes, the flux of the complex reaches a maximum at the elution time of the complex as determined by its effective velocity, but the peak maximum is significantly reduced. In this case, most of the metal is released at the beginning of the column and the peak of "free" metal is observed near its elution time (Fig. 4.4c). If metal adsorption is important, the area of the "free" metal peak will be decreased.

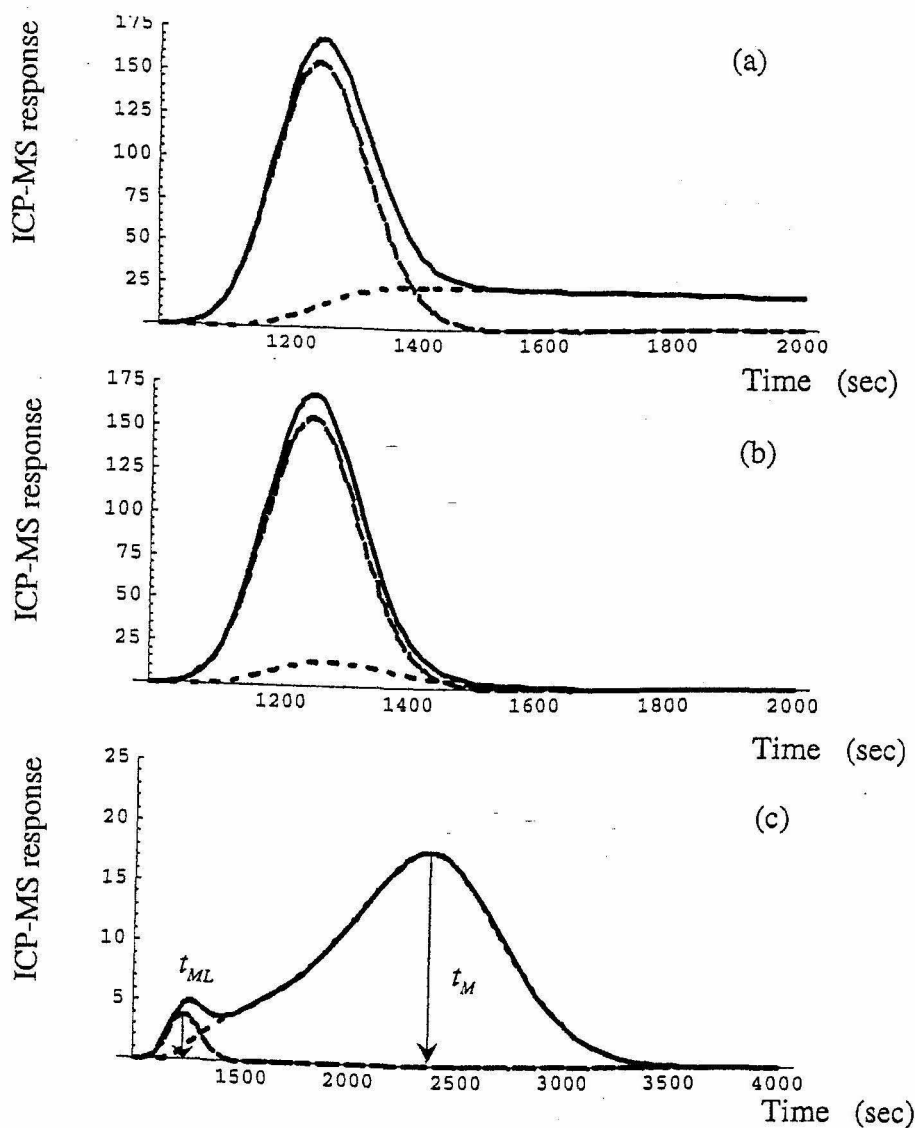


Figure 4.4. Metal complex (— — —), "free" metal (- - -), and total metal (——) fluxes (arbitrary units) vs. time. Values of parameters: $v_M = 0.012$ cm/sec, $v_{ML} = 0.024$ cm/sec, $s_{ML} = 0.05$ cm/(sec)^{1/2}, $s_M = 0.06$ cm/(sec)^{1/2}.

(a) Slow adsorption, quasi-inert complex: $k_b = 0.001$ sec⁻¹, $k_m = 0.00075$ sec⁻¹. Note that due to the slow metal adsorption, a tail of "free" metal is formed.

(b) Fast adsorption, quasi-inert complex: $k_b = 0.001$ sec⁻¹, $k_m = 0.0048$ sec⁻¹. Note that due to the fast adsorptive metal loss, there is no metal tailing as in case (a).

(c) Slow adsorption, labile complex: $k_b = 0.004$ sec⁻¹, $k_m = 0.001$ sec⁻¹. Note that due to the fast dissociation, a separate metal peak is observed at later elution times; because of the metal adsorptive loss the ICP-MS signal is smaller than in the first two cases where most of the metal is present in the form of the complex (different scale on x and y-axes).

4.3 Mathematical description of the system

This qualitative view is given quantitative meaning by writing a system of differential equations for the complexed and "free" metal and by examining the solutions of the system under certain limiting conditions.

4.3.1 Governing equations

In the most general way, the spatial and temporal evolution of the concentrations of the analytes (i.e., the complex, the free ligand and the free metal) are described by a nonlinear system of partial differential equations. If (re)formation of the complex is neglected, this simplifies to a system of linear partial differential equations.

The mass conservation equations for complexed and "free" metal concentrations are given by

$$\frac{\partial ML(x,t)}{\partial t} + v_{ML} \frac{\partial ML(x,t)}{\partial x} = \frac{s_{ML}^2}{2} \frac{\partial^2 ML(x,t)}{\partial x^2} - k_b ML(x,t) \quad (1)$$

$$\frac{\partial M(x,t)}{\partial t} + v_M \frac{\partial M(x,t)}{\partial x} = \frac{s_M^2}{2} \frac{\partial^2 M(x,t)}{\partial x^2} + k_b ML(x,t) - k_m M(x,t) \quad (2)$$

On the left-hand side of Eqs. (1) and (2), the first terms describe changes in the concentrations at a given point and the second terms account for advective transport. The first terms on the right-hand side account for diffusive transport and the remaining

terms describe dissociation of the metal complex and the adsorptive loss of the "free" metal to the column.

A general analytic solution can be found for this system of linear partial differential equations. The exact solution is tractable for Eq. (1) (see section 4.3.2) and an approximate solution is obtained for Eq. (2) (see section 4.3.3 and Appendix 4A). Further approximations are made (in section 4.3.4) that correspond to the diffusion- and advection-dominated regimes described in section 4.2.2. This allows the equilibrium concentration of the complex to be extracted, in some cases, from the experiment elution profiles (see section 4.4).

4.3.2 Concentration profile for the metal complex

The linear, homogeneous Eq. (1) for the concentration of the complexed metal can be solved independently of Eq. (2) for the "free" metal. With the initial condition $ML(x,0) = N \delta(x)$ (where $\delta(x)$ is Dirac's delta function), the solution to equation (1) is given by:

$$ML(x,t) = N \frac{1}{\sqrt{2\pi t s_{ML}}} \exp[-k_b t] \exp\left[-\frac{(x - v_{ML} t)^2}{2s_{ML}^2 t}\right] \quad (3)$$

Here the term $\exp[-k_b t]$ incorporates the effect of the dissociation while the term

$\exp\left[-\frac{(x - v_{ML} t)^2}{2s_{ML}^2 t}\right]$ corresponds to diffusion and advection.

The flux of the metal complex through a cross-section of the chromatographic column is given by the sum of advection and diffusion transport terms:

$$F_{ML}(x,t) = v_{ML}ML(x,t) - \frac{s_{ML}^2}{2} \frac{\partial ML(x,t)}{\partial x} \quad (4)$$

Now the elution time of the maximum flux of the metal complex through the detector will be determined. This will allow the diffusion- and advection-dominated regimes described in section 4.2.2 to be defined quantitatively. After that the analysis of the concentration profile of the "free" metal will be continued, in section 4.3.3.

4.3.2.1 Elution time for the complexed metal

The time dependence of the flux of the complexed metal is mainly governed by the exponential terms in Eq. (3). The elution time (t_{\max}) at which the maximum flux of the complex is observed at the position $x = L$ is given by the solution to the equation $\frac{dF_{ML}(x,t)}{dt} = 0$ for $x = L$. The latter can be estimated by considering only the temporal dependence of the exponents in Eq. (3). Then t_{\max} is given by

$$t_{\max} = \frac{L}{\sqrt{2k_b s_{ML}^2 + v_{ML}^2}} \approx \begin{cases} L/v_{ML} & \text{if } k_b \ll (v_{ML}/s_{ML})^2 \\ L/(s_{ML}\sqrt{2k_b}) & \text{if } k_b \gg (v_{ML}/s_{ML})^2 \end{cases} \quad (5)$$

Eq. (5) corresponds to two general regimes with respect to the time scale of diffusion (i.e., the advection-dominated and diffusion-dominated regimes introduced in section 4.2.2). These regimes are illustrated in Fig. 4.3:

1. The advection-dominated dissociation, $k_b \ll (v_{ML} / s_{ML})^2$. In this case the elution time/volume at which the peak for the complex elutes is determined by its effective velocity (i.e., $t_{\max} \approx \frac{L}{v_{ML}} = t_{ML}$).
2. The diffusion-dominated dissociation, $k_b \gg (v_{ML} / s_{ML})^2$, the elution time/volume at which the peak for the complex elutes, is determined by the balance between the diffusion and the complex dissociation. Under this regime the peak elutes earlier than if its position were determined by the effective velocity of the complex (i.e., $t_{\max} < t_{ML}$). Also, the area of the peak for the complex is smaller than in the case of advection-dominated dissociation, due to the significant metal loss from the complex.

For all the experiments in the present work, the advection-dominated regime of dissociation, has been observed and is discussed from now on.

4.3.3 "Free" metal concentration profile

Given the solution to the Eq. (1) for the concentration of the metal complex the Eq. (2) for the "free" metal ion concentration can be now solved. Eq. (2) is a linear inhomogeneous equation, hence its generic solution is a sum of its arbitrary solution and a solution to the homogeneous equation (which is the Eq. (2) with ML set to zero). However, the initial condition $M(x,0) = 0$ simplifies the solution, which can be expressed through the Green's function, $G_M(x - x', t - t')$, of the corresponding homogeneous

equation (Eq.(2) without the term $k_b ML(x,t)$):

$$M(x,t) = k_b \int_0^t dt' \int dx' G_M(x-x', t-t') ML(x', t') \quad (6)$$

The Green's function of the homogeneous equation is

$$G_M(x-x', t-t') = \frac{1}{\sqrt{2\pi(t-t')} s_M} \exp[-k_b(t-t')] \exp\left[-\frac{(x-v_M(t-t')-x')^2}{2s_M^2(t-t')}\right] \quad (7)$$

Eq. (6) gives the concentration of the "free" metal in the integral form. The analytic approximation of its solution is given in the Appendix 4A.

Eqs. (3) and (6) describe concentration profiles of the complexed and the "free" metal at a given point in space and time. Now these equations can be simplified under certain conditions to obtain mathematical expressions that can be compared against experimental data for advection-dominated dissociation. Many useful relations for the total amount of metal passing through the detector may be obtained from simplified relations when diffusion is neglected both for quasi-inert and labile complexes. The description of the concentration profiles neglecting diffusion is given in the next section. The validity of zero diffusion approximation for the "free" metal is discussed in the Appendix 4A.

4.3.4 Expressions for the total amount of metal (neglecting diffusion)

In this section, the total amount of metal which has eluted from the chromatographic column is computed. Because diffusion does not change the total amount of metal passing through the detector, it can be neglected in obtaining the total metal flux

F_T . In the limit s_{ML} and $s_M \rightarrow 0$, the solution for ML is

$$ML(x,t) = Ne^{-k_b t} \delta(x - v_{ML} t) \quad (8)$$

while the Green's function (Eq. 7) becomes

$$G_M(x - x', t - t')_{s_M=0} = e^{-k_b(t-t')} \delta(x - v_M(t-t') - x') \quad (9)$$

In the absence of diffusion, the complex is localized at a point $x = v_{ML} t$ (Eq. 8). The delta function in Eq. (9) imposes a condition that the "free" metal released at the time t' at the point with the coordinate x' reaches the point with the coordinate x at the time t . The integration of Eq. (6) may be performed using Dirac's delta functions and the expression for the "free" metal concentration then has the following form:

$$M(x,t) = \frac{k_b N}{v_{ML} - v_M} \exp\left[-\left(\frac{x(k_b - k_m)}{v_{ML} - v_M}\right) - \frac{t(v_{ML} k_m - k_b v_M)}{v_{ML} - v_M}\right] \quad (10)$$

where $x/v_{ML} < t < x/v_M$

The total flux of metal passing through any distance x from the injection point is a sum of the metal remaining in the complex, $v_{ML} N \exp[-\frac{k_b x}{v_{ML}}]$, and the "free" metal,

$$v_M \int_{x/v_{ML}}^{x/v_M} dt M(x,t) = \frac{k_b N v_M}{v_{ML} k_m - k_b v_M} \left(\exp\left[-\frac{k_b x}{v_{ML}}\right] - \exp\left[-\frac{k_m x}{v_M}\right] \right) \quad (11)$$

and is given by

$$F_T = \frac{N}{v_{ML} k_m - k_b v_M} \left(v_{ML} k_m \exp\left[-\frac{k_b x}{v_{ML}}\right] - v_M k_b \exp\left[-\frac{k_m x}{v_M}\right] \right) \quad (12)$$

Eq. (12) may be simplified for the following situations, illustrated in Fig. 4.4 and previously described in the section 4.2.2. Note that the equations below represent the total metal flux quantified by areas integrated under the experimental curves.

1. In the case of quasi-inert complex with slow adsorption ($k_b \frac{x}{v_{ML}}, k_m \frac{x}{v_M} \ll 1$ or

$k_b t_{ML}, k_m t_M \ll 1$), Eq. (12) gives

$$F_T \approx N \left(1 - \frac{k_b L^2 k_m}{2 v_{ML} v_M} \right) = N \left(1 - \frac{t_{ML} t_M k_b k_m}{2} \right) \quad (13)$$

The decrease in the total metal flux with distance is proportional to L^2 (or t^2). The metal flux measured by ICP-MS then mostly derives from the complexed metal and the "free" metal contributes mainly to the tailing of the curve (Fig. 4.4 a).

2. In the case of a quasi-inert complex ($k_b \frac{x}{v_{ML}} \ll 1$ or $k_b t_{ML} \ll 1$) with fast ad-

sorption ($k_m \frac{x}{v_M} \geq 1$ or $k_m t_M \geq 1$), the second term of Eq. (12) may be neglected and the

total metal flux is given by

$$F_T = \frac{N}{1 - \frac{k_b v_M}{v_{ML} k_m}} \exp\left[-\frac{k_b L}{v_{ML}}\right] \quad (14)$$

For this case, the main contribution of the "free" metal to the total metal flux measured by ICP-MS should be observed near the peak of the complex. The tailing part of the curve is less influenced by the non-bound metal due to its loss to the column and there is no separately eluting metal peak at later elution times (Fig. 4.4 b).

3. In the case of a labile complex ($k_b \frac{x}{v_{ML}} \geq 1$ or $k_b t_{ML} \geq 1$) with slow adsorption

($k_m \frac{x}{v_M} \ll 1$ or $k_m t_M \ll 1$) the first term of Eq. (12) may be neglected and the total

metal flux is given by

$$F_T = \frac{N}{1 - \frac{k_m v_{ML}}{v_M k_b}} \exp\left[-\frac{k_m L}{v_M}\right] \quad (15)$$

Because of the fast metal complex dissociation, the total metal flux is mostly due to the "free" metal dissociated from the complex. If loss of "free" metal to the column is slow (slow adsorption), two separate peaks for the metal (i.e., as the complex and as "free" metal released from the complex) may be observed (Fig. 4.4 c).

4.4 Experimental determination of rate constant for dissociation and the equilibrium concentration of the metal complex

Now the formulae obtained can be applied to extract the rate constants for dissociation and/or equilibrium concentration of the metal complex from the experimental data. It is useful to rewrite Eq. (10) for the "free" metal concentration at a given point (x, t), in terms of the following two new parameters:

$$\Delta_{ML} = t_M - x/v_{ML} \quad \text{and} \quad \Delta_M = x/v_M - t_M$$

where Δ_{ML} is the difference between the elution time for the "free" metal released from the complex and the elution time of the peak of the complex ML and Δ_M is the difference between the elution time for the peak of the "free" metal released from the complex and that for the "free" metal in the absence of the ligand. Then Eq. (10) takes the form

$$M(x,t) = \frac{k_b N}{v_{ML} - v_M} \exp\left[-\frac{k_b v_M \Delta_M}{v_{ML} - v_M} - \frac{k_m v_{ML} \Delta_{ML}}{v_{ML} - v_M}\right] \quad (16)$$

The expression suggests some practical ways of estimating kinetic parameters for a metal complex undergoing dissociation.

In the case of a labile complex with slow adsorption (case 3 in section 4.3.4), a separate peak of $M(x,t)$ appears near the elution time of peak for the "free" metal in the absence of the ligand, that is at $t = L/v_M$, where $\Delta_{ML} = L/v_M - L/v_{ML}$ and

$\Delta_M \ll \Delta_{ML}$. Then

$$k_m \geq \frac{v_M}{L} \text{ and } k_b \cong \frac{v_{ML} - v_M}{v_M \Delta_M} \quad (17)$$

Eq. (17) suggests that the rate constant for dissociation may be directly estimated from the position of peak for the "free" metal released from the dissociating complex. This situation is observed for the NiEDDHA complex (Fig. 4.1), for which the elution volume corresponding to the elution time of Ni released from the complex (15.9 mL) is close to the elution volume of "free" Ni injected on the column in the absence of the ligand (16.2 mL). The effective velocities of the complex ($v_{ML} = 1.29$ cm/min) and the metal ($v_M = 0.74$ cm/min) may be estimated from the elution volumes of the corresponding peak maxima (9.3 and 16.2 mL respectively) at a known flow rate (0.4 mL/min) for the column length of 30 cm. The parameter Δ_M is given by the difference between the elution times for the "free" metal released from the complex and in the absence of the ligand, that is: $(16.2 - 15.9)/0.4 = 0.75$ [min]. Then from Eq. (17) the dissociation rate constant for NiEDDHA is estimated to be approximately 0.02 sec^{-1} .

For other limiting regimes, when the dissociation rate constant can not be estimated directly, one of the simplified equations for the total metal flux (Eqs. 14 or 15) may be used. Both k_b and the equilibrium concentration of the metal complex (N) may be estimated in the case of a quasi-inert complex with fast adsorption (Eq. (14)).

In that case (case 2 in section 4.3.4) the peak of the "free" metal, M, appears near the peak of the complex, that is at $t = L/v_{ML}$, where $\Delta_M \cong L/v_M - L/v_{ML}$, and $\Delta_{ML} \ll \Delta_M$. Then

$$k_b \geq \frac{v_M}{L} \text{ and } k_m \equiv \frac{v_{ML} - v_M}{v_{ML} \Delta_{ML}} \quad (18)$$

In the case of a quasi-inert complex with slow adsorption (case 1 in section 4.3.4; Eq. (13)) N may be estimated. This may be done by varying the flow rate and thus the elution time of the complex, plotting the measured metal concentration vs. elution time and fitting the experimental data. If the obtained fit is an exponential, $ML(t) = ae^{-bt}$ (for $k_m \geq v_M / x$; quasi-inert complex, fast adsorption) then k_m may be estimated from Eq. (18) and k_b and N from Eq. (14) ($k_b = b$ and $N = a(1 - \frac{k_m v_{ML}}{v_M k_b})$). If the fit obtained is a second order polynomial, $ML(t) = a + bt + ct^2$ (for $k_b, k_m \ll x / v_{ML}$; quasi-inert complex, slow adsorption), then N may be estimated from Eq. (13) ($N = a$).

This situation is illustrated by experiments with the kinetically-labile CuNTA complexes. The ICP-MS chromatogram of this complex shows a wide Cu peak corresponding to the metal remaining in the complex and released metal (Fig. 4.5). The positions of the peak maxima for the metal complex and "free" metal obtained from such chromatograms are somewhat arbitrary. Nevertheless, a rough estimate of the rate constant for dissociation and the equilibrium concentration of the metal complex may be obtained. For $v_{ML} = 1.43$ cm/min, $v_M = 0.74$ cm/min and $\Delta_{ML} \equiv 100 \pm 25$ sec, the adsorption rate constant is estimated to be $0.005 \pm 0.001 \text{ sec}^{-1}$ (Eq. (18)). Data from experiments with varying flow rates (and thus elution times) (Fig. 4.6) can be fit with an exponential expression, $ML = (5 \times 10^{-6})e^{-0.002t_{ML}}$.

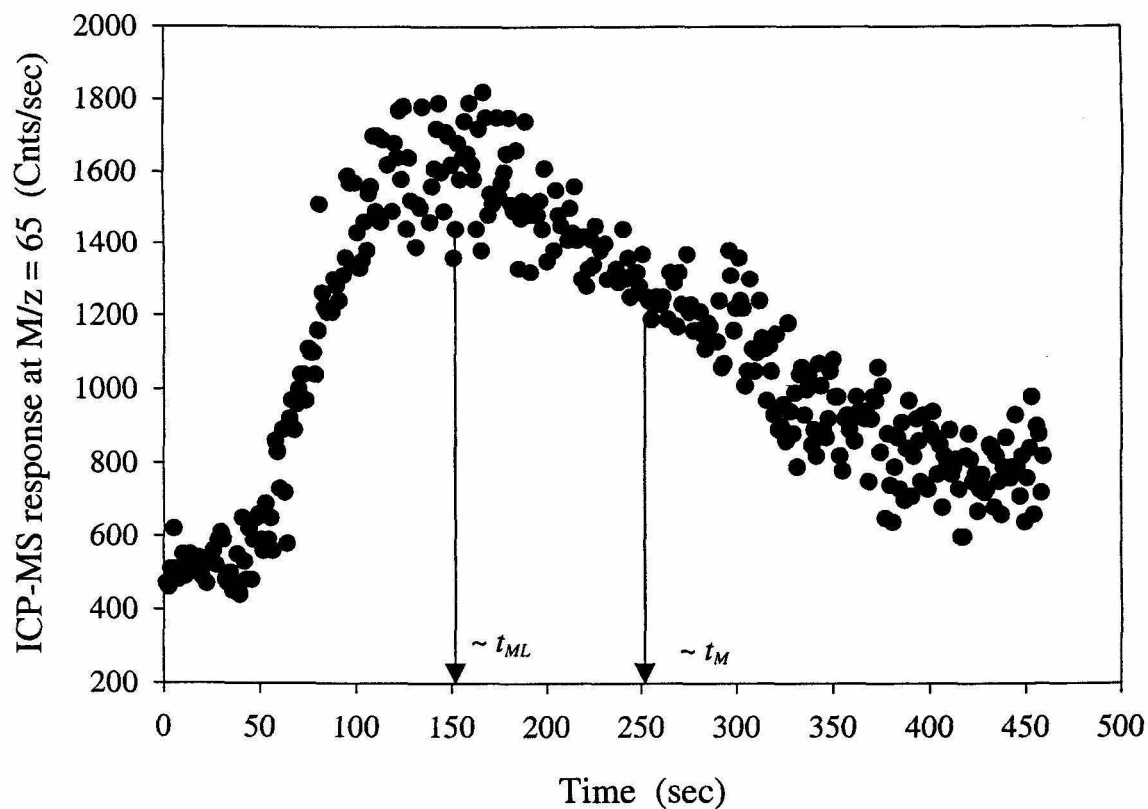


Figure 4.5. ICP-MS chromatogram obtained with ICP-MS for $[\text{CuNTA}]_0 = 4 \mu\text{M}$ eluting from a chromatographic column (TSKGEL 3000 PW), ionic strength - 10 mM (ammonium acetate), pH = 6. Approximate elution times corresponding to the maxima of the metal complex and "free" metal are shown by t_{ML} and t_M respectively.

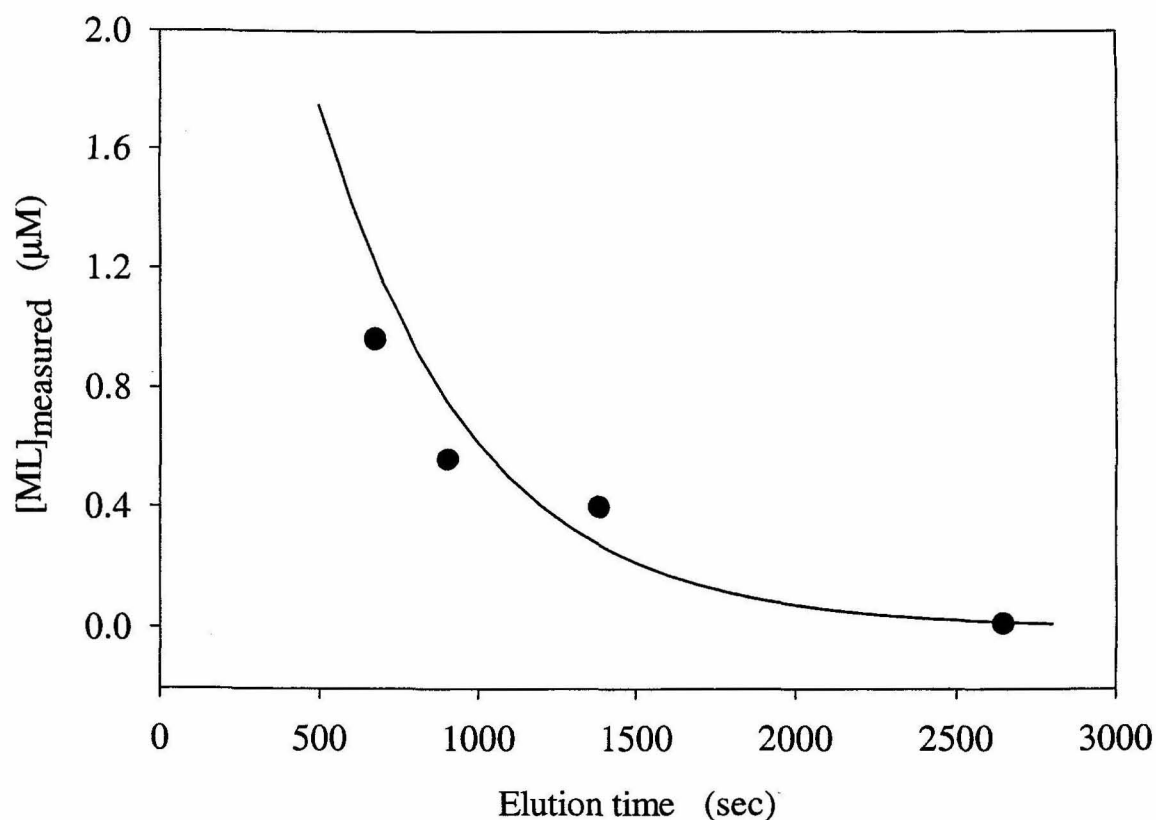


Figure 4.6. Dependence of CuNTA concentration eluting from a chromatographic column (TSKGEL 3000 PW) on the elution time of the peak maximum for the complex, t_{ML} , (varied flow rates). Conditions: $[\text{CuNTA}]_0 = 4 \mu\text{M}$, ionic strength - 10 mM (ammonium acetate), pH = 6. Experimental data are fitted with the curve: $y = 5 \times 10^{-6} \exp[-0.002 \cdot t]$.

The fitting parameters provide an estimate of the rate constant for dissociation, $k_b \approx 0.002 \text{ sec}^{-1}$, and show relatively good agreement between the known concentration of the complex in the initial solution ($4.0 \times 10^{-6} \text{ M}$) and that calculated from the expression $N = a(1 - \frac{k_m v_{ML}}{v_M k_b}) = 3.9 \pm 0.3 \times 10^{-6} \text{ M}$. The value estimated for the rate constant for dissociation of the CuNTA complex is in good agreement with the value of $(2 \pm 1) \times 10^{-3} \text{ sec}^{-1}$ determined from ligand-exchange experiments with CuNTA at pH 7.4 (Hering and Morel, 1990).

It is important to note that the extent of metal - matrix interactions, described in the model by the adsorption rate constant (k_m) and assumed to be constant, can be dependent on the flow rate and may vary in the experiments described leading to additional uncertainties in the values determined for k_b and N .

This approach, although time consuming, may be also helpful in determining if the equilibrium speciation of the sample has been altered by the non-equilibrium conditions of the chromatographic separation. This may be especially useful in the case of redistribution of the metal between different species without loss of the metal to the column. In this case, since the mass balance of the metal is preserved, complex dissociation would be difficult to detect. It may be expected that, for kinetically-labile species, the distribution of the metal between different fractions will vary with the flow rate.

Another approach to estimation of the kinetic characteristics and equilibrium concentrations of a metal complex is the numerical modeling of the total metal concentration profiles obtained with a metal detection technique. However, the expression for the total metal flux (Eq. (12)) is rather complicated with many fitting parameters, most of which

appear in the exponentials. In addition, the numerical modeling of experimental curves would necessarily involve general differential equations that include diffusion terms for the metal complex and "free" metal. Attempts to do such modeling of concentration profiles for the metal complex (without taking the "free" metal into account) have demonstrated that the system is not sufficiently constrained; the system is also unstable with respect to small perturbations and as a consequence the parameters of interest are difficult to obtain.

4.5 Concluding remarks

The characteristics of metal-organic complexes govern the bioavailability and toxicity of metals. Coupled chromatographic metal detection techniques are applicable for studying kinetically-inert complexes as shown in Chapter 3. But these methods can not be directly applied to kinetically-labile complexes. This chapter provides an extension of the method to include kinetically-labile complexes under some conditions.

A mathematical model of the chromatographic behavior of the metal complex and the "free" metal has been developed. The model takes into account several processes which occur during the chromatographic separation: dissociation of the metal complex, diffusion, and adsorption of the "free" metal onto the column. This model allows the estimation of rate constant for complex dissociation and the (initial) equilibrium concentration of complexes that are partially labile under chromatographic conditions. Although the results of this modeling were obtained for a coupled SEC/ICP-MS system, they are generally applicable to all chromatographic techniques used for metal complexation

studies that involve separation of the complex from the metal and/or ligand and thus perturb equilibrium conditions.

Acknowledgements

I am grateful to Maxim Lyutikov, John Brady, Nikita Nekrasov, Peter Green, and Zhen-Gang Wang for their help, useful comments and discussions.

APPENDIX 4A

CONCENTRATION PROFILES OF THE "FREE" METAL FLUX (INCLUDING EFFECTS OF DIFFUSION)

The "free" metal concentration may be obtained by integrating Eq. (6) with the Green's function and ML concentration given by Eqs. (7) and (3) respectively:

$$M(x,t) = N \int_0^t dt' \frac{\sqrt{2\pi}}{\sqrt{s_{ML}^2 t' + (t-t')s_M^2}} \exp[-(k_b t') - (t-t')k_m] e^{-U(t')} \quad (19)$$

where

$$U(t') = \frac{(t'v_{ML} - x + (t-t')v_M)^2}{2(s_{ML}^2 t' + (t-t')s_M^2)} \quad (20)$$

The above integral can be estimated by the saddle point method. The factor in front of the exponent and the first exponent (containing k_b and k_m) are approximately constant and the integral may be evaluated near the maximum of the second exponent. The argument of the second exponent itself may be expanded near the maximum up to the second order in $t' - t'_{\max}$ (the first order is zero near the maximum). Differentiating U with respect to t' (time of the metal release from the complex) and equating it to zero gives the location of the maximum (note that $U(t') \geq 0$ and $U(t'_{\max}) = 0$):

$$t'_{\max} = \frac{x - tv_M}{v_{ML} - v_M} \quad (21)$$

For the saddle point to lie within the integration limits, $0 < t'_{\max} < t$, the following condition must be met

$0 < \frac{x - tv_M}{v_{ML} - v_M}$ and $\frac{x - tv_M}{v_{ML} - v_M} < t$. For $v_{ML} > v_M$, this simplifies to the

two inequalities: $t < \frac{x}{v_M}$ and $\frac{x}{v_{ML}} < t$.

The inequality $\frac{x}{v_{ML}} < t$ corresponds to the negligible release of the metal from the

complex before the elution of the peak maximum for the complex. This is reasonable

since the concentration of ML eluting before the peak is small. The inequality $t < \frac{x}{v_M}$

states that, for $t > \frac{L}{v_M}$, all metal released from the complex has passed through the de-

tector (note that $\frac{L}{v_M}$ is the maximum time that metal released at the beginning of the col-

umn would take to reach the detector). Since $U(t' = t'_{\max}) = 0$ and

$$\frac{\partial^2 U}{\partial t'^2} \Big|_{t'=t'_{\max}} = \frac{(v - v_M)^3}{(tv_{ML} - x)s_M^2 + s_{ML}^2(x - tv_M)} \quad (22)$$

it can be found that

$$M(x, t) = \frac{N\sqrt{v_{ML} - v_M}}{\sqrt{2\pi}\sqrt{(tv_{ML} - x)s_M^2 + (x - tv_M)s_{ML}^2}} \exp\left[-\frac{(tv_{ML} - x)k_m + k_b(x - tv_M)}{v_{ML} - v_M}\right] \times \int_0^t dt' \exp\left[-\frac{1}{2} \frac{\partial^2 U}{\partial t'^2} \Big|_{t'=t'_{\max}} (t' - t'_{\max})^2\right] \quad (23)$$

After the integration we obtain:

$$M(x,t) = \frac{N}{2(v_{ML} - v_M)^2} \exp\left[-\left(\frac{x(k_b - k_m)}{v_{ML} - v_M}\right) - \frac{t(v_{ML}k_m - k_b v_M)}{v_{ML} - v_M}\right] \\ \times \left(Erf\left(\frac{(tv_{ML} - x)\sqrt{v_{ML} - v_M}}{s_t}\right) + Erf\left(\frac{(x - tv_M)\sqrt{v_{ML} - v_M}}{s_t}\right)\right) \quad (24)$$

where Erf is an error function and $s_t^2 = 2(tv_{ML} - x)s_M^2 + s^2(x - tv_M)$ is a cumulative diffusion.

Eq. (24) may be simplified if diffusion can be neglected either for the "free" or complexed metal:

If $s_{ML} = 0$,

$$M(x,t) = \frac{k_b N}{2(v_{ML} - v_M)} \exp\left[-\left(\frac{x(k_b - k_m)}{v_{ML} - v_M}\right) - \frac{t(v_{ML}k_m - k_b v_M)}{v_{ML} - v_M}\right] \\ \times \left(Erf\left(\frac{\sqrt{(tv_{ML} - x)\sqrt{v_{ML} - v_M}}}{\sqrt{2}s_M}\right) + Erf\left(\frac{(x - tv_M)\sqrt{v_{ML} - v_M}}{\sqrt{tv_{ML} - x}\sqrt{2}s_M}\right)\right)$$

If $s_M = 0$,

$$M(x,t) = \frac{k_b N}{2(v_{ML} - v_M)} \exp\left[-\left(\frac{x(k_b - k_m)}{v_{ML} - v_M}\right) - \frac{t(v_{ML}k_m - k_b v_M)}{v - v_M}\right] \\ \times \left(Erf\left(\frac{(tv_{ML} - x)\sqrt{v_{ML} - v_M}}{\sqrt{x - tv_M}\sqrt{2}s_{ML}}\right) + Erf\left(\frac{\sqrt{(x - tv_M)\sqrt{v_{ML} - v_M}}}{\sqrt{2}s_{ML}}\right)\right) \quad (25)$$

In the limit when both s_M and s_{ML} are equal to zero, Eq. (25) reduces to Eq. (10). The total metal flux through a detector is given by the sum of the fluxes of the "free" metal (Eq. 25) and the metal complex (Eq. 3).

On inspection of Eq. 24, it may be concluded that, for small s_{ML} and s_M , the error functions may be approximated by unity (i.e., diffusion may be neglected) everywhere

except near the elution time of the peak maxima for metal complex (t_{ML}) and "free" metal (t_M).

Chapter 5

STUDIES OF COPPER-HUMIC INTERACTIONS USING SEC/ICP-MS

5.1 Introduction

Humic substances are natural organic polyelectrolytes that are formed in biogeochemical weathering of plant and animal remains. They are made up of humic acids, which are insoluble in water under acidic conditions ($\text{pH} < 2$), fulvic acids which dissolve under all pH conditions and have lower molecular weights than humic acids, and humins, which are insoluble under all pH conditions. The major functional groups of humic materials include carboxylic acid, phenolic and alcoholic hydroxyls, ketone, and quinone groups (Davies and Ghabbour, 1999). Fulvic acids are somewhat more aliphatic and less aromatic than humic acids (Gaffney et al. 1996 and ref. cited). The structure of humic materials allows them to function as surfactants, with the ability to bind both hydrophobic and hydrophilic compounds. Thus humic and fulvic acids are effective agents in transporting both organic and inorganic constituents, such as organic pollutants and metals, in natural waters.

Since humic substances are ubiquitous and often are present in estuaries at concentrations greater than 1 mg/L they have, for a long time, been recognized as weak

metal ligands (Buffle, 1988). They have been shown to play an important role in metal complexation in systems with high dissolved organic carbon (DOC) concentrations and relatively high metal concentrations (Breault et al., 1996). However, recent studies conducted at low copper concentrations have suggested that humic and fulvic acids also can form relatively strong complexes with Cu (Xue and Sigg, 1999; Kogut and Voelker, 2000).

The goal of this study was to investigate strong copper-humic interactions using SEC/ICP-MS method described in previous chapters (see Chapters 3 and 4). The work was performed with Suwannee River humic acid, which is commonly used as a reference humic acid.

5.2 Background

5.2.1 Sources and composition of humics

Humic acids are complex aliphatic-aromatic macromolecules which in addition to the main functional groups have been shown to contain bound polysaccharides, proteins and peptides (Watt et al. 1996, Fan et al., 2000).

Studies of humic substances with the high-resolution X-ray spectromicroscopic method have shown that their macromolecular structures (size and shape) vary with the origin (soil versus fluvial), solution chemistry, and associated mineralogy (Myneni et al., 2000). Characterization of sources and speciation of aquatic fulvic acids using

fluorescence measurements have demonstrated that their composition have seasonal variations correlated with biological productivity (McKnight et al., 2000).

The analysis of humic acids is complicated because they are complex mixtures of organic substances with a wide range of molecular sizes that also can form secondary micelle-like structures (Engelbrektson and von Wandruszka, 1994). Molecular weight, size, and shape of humic acids have been studied with ultracentrifugation, viscosity measurements, cryoscopy, light scattering, electrophoresis and size exclusion chromatography (SEC) (Hayes et al., 1989 and ref. cited). SEC has been widely used to separate humic acids into size fractions (DeNobili and Chen, 1999 and ref. cited). Problems can arise during this separation due to the tendency of humic acids to aggregate/disaggregate as the result of their interactions with the mobile and stationary phases. It has been suggested that these interactions may disrupt a micelle-like conformation of humic acids (Piccolo et al., 1996). However, a later study of humic acids with SEC in the presence of organic or inorganic acids indicated that the observed shift in retention time results mainly from hydrophobic interactions of humic substances with the stationary phase and not from disruption of micelle-like structures (Varga et al., 2000). This implies that, for characterization of humic substances by size-exclusion chromatography, ionic and hydrophobic interactions with the stationary phase must be considered. Reported molecular weight of humic acids varies significantly depending on the method and conditions under which it was determined. The values in the range between 3 and 50 kDa for aquatic humic substances and up to 500 kDa for soil humic substances have been reported (Gaffney et al. 1996 and ref. cited).

5.2.2 Copper complexation by humic and fulvic acids

Metal complexation by isolated fulvic and humic acids has been extensively studied (Xue and Sigg, 1999 and ref. cited). Most of the literature data on Cu-binding by humic and fulvic acids have been obtained under higher metal concentrations than those typically encountered in natural waters. EXAFS studies of copper-humic interactions performed with copper-loaded water samples show no interaction between the bound metal centers and indicate that each copper is bound by four equatorial and two more distant axial oxygen atoms in a distorted octahedral geometry (Korshin et al., 1998). It has been suggested, that at elevated metal concentrations, copper is bound with the clusters of acidic functional groups, mostly carboxylic, in both humic and fulvic acids (Leenheer et al., 1998; Davies et al., 1997).

Conditional stability constants for Cu with humic and fulvic acids have been determined by metal titration experiments. Most commonly, these have been performed using ion selective electrodes at elevated metal concentrations. The conditional stability constants reported (for pH 6) are on the order of $\log K_{\text{CuL}}^{\text{cond}} < 10$ for the strongest sites and $\log K_{\text{CuL}}^{\text{cond}} = 5.5$ for the majority of the sites indicating formation of relatively weak complexes (Leenheer et al., 1998). At lower free cupric ion concentrations ($\{\text{Cu}^{2+}\} < 10^{-6}$ M), the contribution of phenolic sites to copper binding becomes increasingly important at pH 6 (Benedetti et al., 1995). Copper complexation by phenolic and carboxylic groups of humic and fulvic acids can be important at elevated total copper concentrations. Fulvic acid was found to be a major constituent of the natural ligands complexing copper

in the case of streams with relatively high copper concentration and low pH (Breault et al., 1996). Literature data on copper titrations of whole water samples collected from southeastern U.S. rivers and a pond in Fontainebleau Forest (with $[Cu]_T = 10^{-4} - 10^{-7}$ M) could be successfully modeled by assuming some fraction (50%) of the dissolved organic matter to have the same metal-binding properties as Suwannee River fulvic acid thus suggesting that fulvic acids contribute significantly to metal complexation in natural waters (Cabaniss and Shuman, 1988).

Interactions of humic and fulvic acids with copper at low concentrations, which are relevant to natural water, have recently been studied using electrochemical methods (Xue and Sigg, 1999; Rozan and Benoit, 1999; Kogut and Voelker, 2000).

Xue and Sigg (1999) compared copper complexation by Suwannee River fulvic and purified peat humic acids with whole lake water samples using competitive ligand-exchange and differential pulse cathodic stripping voltammetry (CLE-CSV). They concluded that highly specific copper binding ligands are either not extracted by the usual methods for isolation of humic and fulvic acids or their structures are changed during the extraction procedure. Comparison of the copper titration curves for Suwannee River fulvic and for purified peat humic acids with those for whole lake water samples suggested that ligands present at low concentrations in lake water samples are stronger than the isolated fulvic and humic acids. Reported conditional stability constants and total ligand concentrations are listed in Table 5.1. These stronger ligands were formed in eutrophic lake waters, whereas in a lake with higher metal concentrations and low biological productivity the copper titration curves resembled those for the fulvic acid.

Table 5.1. Reported conditional stability constants and total ligand concentrations for copper titrations of humic and fulvic acids and whole water samples

Sample	Titration conditions				Fitting parameters	
	pH	Ionic strength (M)	[HA] _T (mg/L)	[DOC] (mg/L)	$\log K_{CuL}^{cond}$	[L] _T , (nM)
SRFA ^a	7.5	0.016 M	6.0	3.23	13.3	27.3
	8.0	0.016 M	6.0	3.23	14.1	15.3
peat HA ^a	7.5	0.015	1.32	0.69	12.2	30.1
	7.8	0.015	1.32	0.69	12.5	30.0
lake water ^a	7.8	0.006	N/D	2.8	15.5	30.0
	7.8	0.006	N/D	2.8	13.8	70.0
	7.8	0.006	N/D	1.2	14.3	12.9
SRHA ^b	8.2	0.7	1.0	N/D	12.0	10.4

a - Xue and Sigg, 1999;

b - Kogut and Voelker, 2000.

Symbols: SRFA - Suwannee River fulvic acid; HA - humic acids; SRHA - Suwannee River humic acid; DOC - dissolved organic carbon; N/D - parameter was not determined.

Kogut and Voelker (2000) performed similar studies with Suwannee River humic acid in seawater and found that copper complexation in that system was similar to that observed for the peat humic acid in lake water (Table 5.1).

These recently-reported conditional stability constants are several orders of magnitude higher than those obtained earlier at elevated copper concentrations. Because of their low concentrations, these strong ligands in humic and fulvic acids would be completely titrated at free copper ion concentrations $> 10^{-12}$ M (Xue and Sigg, 1999). Since Cu forms relatively weak complexes with carboxylic functional groups of humic and fulvic acids ($\log K_{CuL}^{cond} = 5.5$, Leenheer et al., 1998), ligands with a high affinity for

copper are likely to involve nitrogen- or sulfur-containing functional groups of amino acids and peptides that are minor constituents of humic and fulvic acids. The peptides that have been shown to be incorporated into the structures of humic acids are at least partially destroyed during the usual isolation procedures (Fan et al., 2000). The importance of reduced sulfur functional groups (thiol and disulfide) has been demonstrated in X-ray absorption spectroscopy studies of Hg(II) complexation by soil humic acids (Xia et al., 1999).

5.3 Materials and methods

5.3.1 Reagents

Eluants for chromatography (10 mM ammonium acetate at pH 6) were prepared from ammonium hydroxide (21.7 vol. %; Seastar) and acetic acid (99.7 vol. %; Seastar) by titration of acetic acid with ammonium hydroxide up to pH 6 and dilution with ultrapure water (18 MΩ cm, Millipore Milli-Q system). Stock solutions and standards were prepared gravimetrically from 1.00 g/L Cu reference solution (Fisher Scientific), ethylenediiminobis (2-hydroxyphenyl) acetic acid (EDDHA, 97%, Aldrich), or Suwannee River humic acid obtained from the International Humic Substance Society (IHSS) using the eluant as a background electrolyte. The elemental composition of the Suwannee River humic acid provided by IHSS is given in Table 5.2.

Table 5.2. Elemental analysis data for Suwannee River humic acid	
Element	Concentration (%)
C	52.89
H	4.14
O	43.40
N	1.17
S	0.58
P	<0.01

Solutions of 50, 100, and 500 mg/L humic acids were prepared gravimetrically in the eluant. These concentrations are significantly higher than those typically encountered in natural waters but were chosen based on the method sensitivity and anticipated concentration of strong copper-binding sites (see section 5.4.2). Solutions of copper and humic acids were pre-equilibrated for three days before the SEC/ICP-MS experiments were performed. In competitive equilibration experiments, the competing ligand, EDDHA, was added to the pre-equilibrated solutions of Cu-humic acids and left for four more days before the SEC/ICP-MS experiments.

The standard tuning solution for ICP-MS (0.50 mg/L of Li, Y, Tl) was prepared from commercially available 1.00 mg/L solutions of these elements (ULTRA Scientific). The internal standard solution was prepared from a 10.00 g/L Ga standard solution (ULTRA Scientific) by dilution in 25% (v/v) nitric acid (Seastar, Canada).

5.3.2 Instrumentation

All the experiments were performed on a coupled SEC/ICP-MS system described in Chapter 3. An additional valve (Pharmacia Biotech, motor valve V - 7) was used for in-line sample introduction to by-pass the column. Results of column calibration and

characterization of ionic and hydrophobic interactions with the column stationary phase are described in Chapter 3. The column cleaning procedure and calibration of ICP-MS response for copper concentration are also given in Chapter 3.

ICP-MS operational parameters for measurements of Cu concentrations in solutions with high concentrations of humic acid are given in Table 5.3.

Table 5.3. ICP-MS operational parameters for samples with high concentration of humic acid	
Parameter	Operational range
Sample depth	6.8 to 7.1 mm
Extraction lenses #1 #2	-180 to -150 -60 to -30
Einzel #2	-5 to 5

5.4 Results and discussion

5.4.1 Chromatographic behavior of humic acids

The chromatographic behavior of humic acids is determined by the structures of the humic acids and their interactions with the stationary phase of the column. Based on the results of column characterization experiments (see Chapter 3) and literature data (DeNobili and Chen, 1999 and ref. cited), charge-exclusion and hydrophobic interactions between the humic acids and the column stationary phase can be expected. Both of these effects will depend on the degree of the humic acids deprotonation, which leads to a

higher negative charge on the humic acids at higher pH values. Since the column has a residual negative charge, charge-exclusion effects will be stronger at higher pH and a larger apparent molecular weight will be calculated based on elution volume. The reported pK_a s of humic acids are on the order of 3 - 4 and 9 (Benedetti, 1995; Kinniburgh, 1996), so the humic acids are partially deprotonated at the experimental pH values of 6 and 7.

Hydrophobic interactions with the stationary phase can be expected to be stronger for the protonated humic acids and dependent on the polarity of the elution solution with decreased hydrophobic interactions for more polar elution solutions. It has been shown that hydrophobic interactions are less important for large molecules than for small molecules (see Chapter 3).

The UV chromatograms of a 500 mg/L solution of humic acid obtained at two different pH values and eluant compositions are shown in Fig 5.1. The UV-absorbing fraction of humic acid elutes as a single broad peak without any significant tailing. The chromatograms are identical within the experimental error for the two pH values and are slightly shifted for the eluant containing 10% methanol. The elution volume (or time) of the humic acids corresponds to an average molecular weight value of 17.2 kDa based on the column calibration with polystyrene sulfonate standards (see Chapter 3). This value is in the range of those reported for Suwannee River natural organic matter (Wagoner and Christman, 1997).

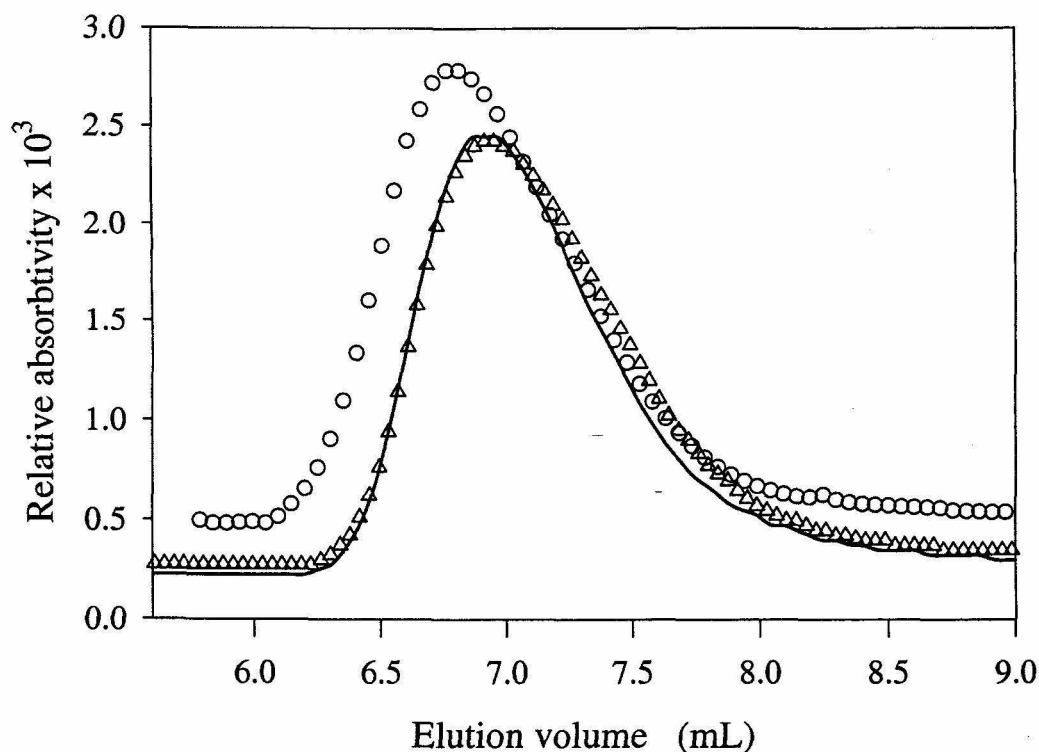


Figure 5.1. UV chromatograms of a 500 mg/L solution of HA.
 Conditions: 10 mM ammonium acetate pH = 6 (—), pH = 7 (Δ);
 10 mM ammonium acetate pH = 6 and 10 % methanol (\circ).

The molecular weight could be underestimated due to the hydrophobic interactions of humic acids with the column stationary phase that would increase the elution volume (or time) of the analyte. The decreased elution volume of the humic acid peak with the addition of 10% methanol to the eluant suggests that these interactions are present. However, as expected for a large molecule, the observed retention shift is not large. The elution volume of the humic acids with 10% methanol corresponds to an average molecular weight value of 21.5 kDa.

Within the narrow pH range examined, no change in the humic acid peak elution volume was observed which suggests that the charge-exclusion interactions are the same

at pH 6 and 7. Charge-exclusion interactions arise from repulsion between the charged analyte and the stationary phase. Since charge exclusion prevents analyte adsorption onto the stationary phase and such adsorption is the bases of hydrophobic interactions, the observed effect of methanol on the elution volume of the humic acids also implies that charge-exclusion interactions are not strong.

Significant charge-exclusion interactions between the partly deprotonated EDTA and the negatively charged column stationary phase result in similar elution volumes for EDTA (molecular weight 336 Da) and humic acids (6.8 mL vs. 7.0 mL). As a result their peaks are not fully resolved. The peaks for humic acids and EDDHA are fully separated (Fig. 5.2) which allowed EDDHA to be used as a competing ligand for the study of copper-humic interactions (see section 5.4.3).

5.4.2 Concentration of strong copper-binding sites in humic acids

The copper concentration in the humic acids was determined by the method of standard additions using direct sample injections into the ICP-MS (i.e., bypassing the column). In this method, systematic additions of Cu were made to samples containing 50 or 100 mg/L humic acids and the observed Cu concentrations were back extrapolated to the initial Cu concentration with no added Cu. Copper was added at concentrations ranging from 0.3 to 1.0×10^{-7} M. The concentration of Cu determined in four replicate experiments was $(2.0 \pm 0.5) \times 10^{-8}$ M in 50 mg/L humic acids and $(3.5 \pm 0.6) \times 10^{-8}$ M in 100 mg/L humic acids.

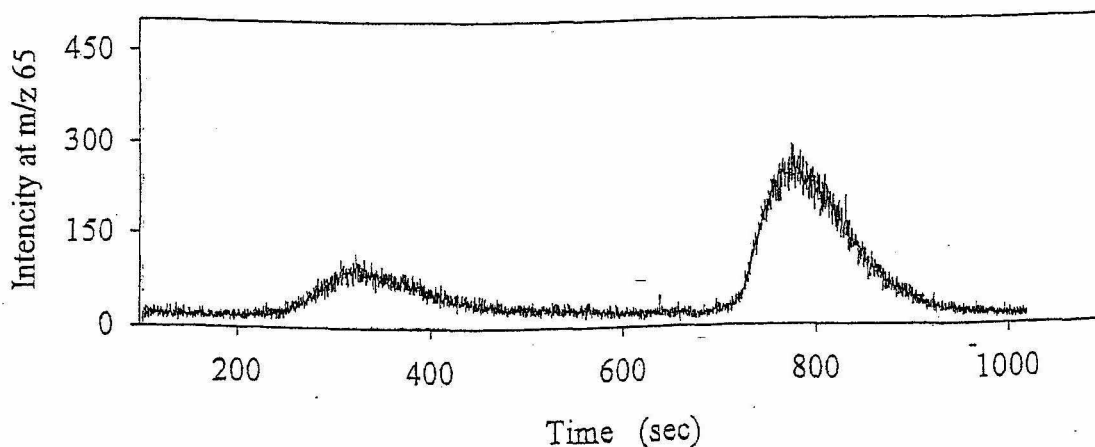


Figure 5.2. ICP-MS chromatogram of Cu-humic acid (CuHA) and CuEDDHA after the separation using SEC. The first peak corresponds to the CuHA complex and the second one to the CuEDDHA complex. Conditions: $[\text{Cu}]_{\text{T}} = 5 \times 10^{-8} \text{ M}$, $[\text{HA}]_{\text{T}} = 50 \text{ mg/L}$, $[\text{EDDHA}]_{\text{T}} = 1.0 \times 10^{-7} \text{ M}$; pH = 6, 10 mM ammonium acetate.

To verify that the samples are not contaminated with Cu during the chromatographic separation, the concentration of Cu in the humic acids was also determined after elution of the sample from the column. The concentrations obtained in eight replicate experiments, $(2.0 \pm 0.6) \times 10^{-8} \text{ M}$ for 50 mg/L humic acids and $(3.8 \pm 0.7) \times 10^{-8}$ for 100 mg/L humic acids, are in good agreement with that determined by-passing column. However, significant experimental error is associated with insufficient sensitivity of the technique at such low Cu concentrations. Matrix effects at high humic acid concentrations cause additional complications.

These results indicate that there is no contamination of the sample or loss of copper from it during the chromatographic separation. Previous work (presented in Chapter 3) has shown that complexes such as CuNTA ($\log K_{ML}^{cond} = 9.6$) are kinetically labile on the column. The retention of Cu in the complex during chromatography suggests that at least some Cu binding sites in the humic acids are stronger than NTA.

As increasing concentrations of Cu were added to the humic acid samples, the concentration of Cu-humic acids (CuHA) eluting from the column also increased (Fig. 5.3). However, the recovery of copper from the column was less than 90% for Cu additions comparable to the ambient Cu concentration in the humic acid sample. Due to the difficulties associated with measuring low Cu concentrations, only a limited number of experiments was performed. Thus only a very rough estimate of Cu-binding parameters for humic acids could be obtained from experimental data.

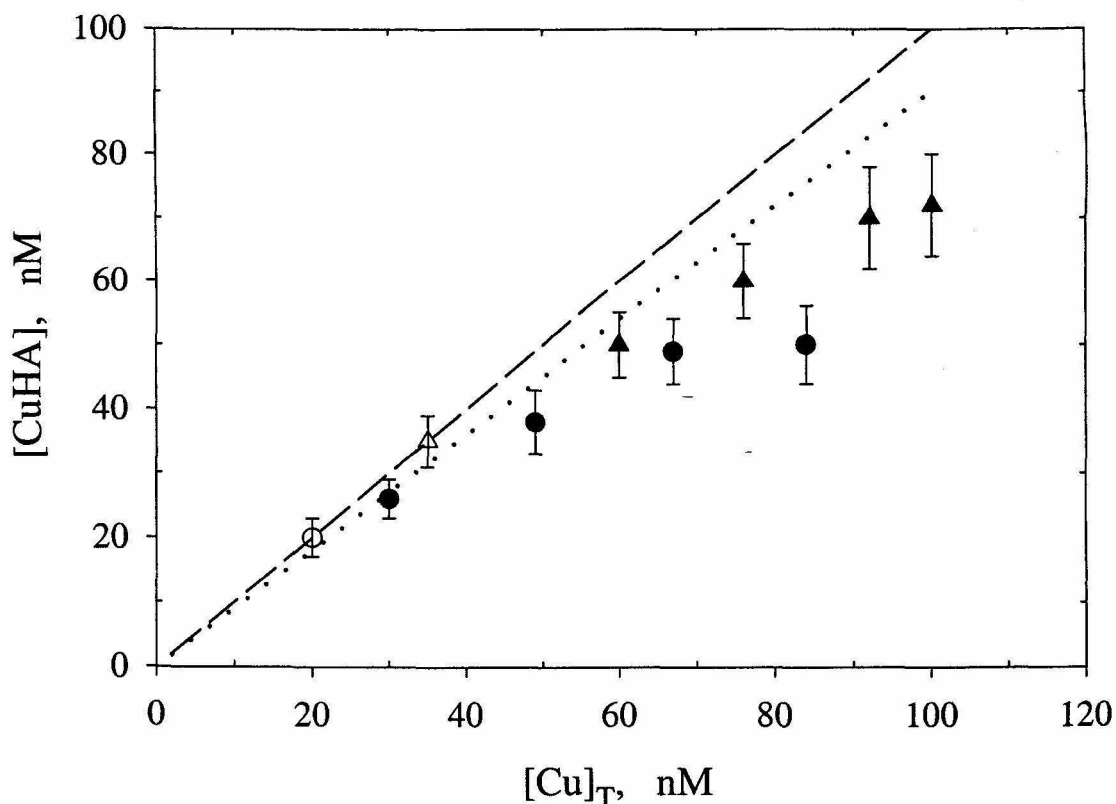


Figure 5.3. Dependence of Cu recovery from the SEC column as measured with ICP MS in CuHA peak on the total Cu concentration. Conditions: 10 mM ammonium acetate, pH = 6. $[HA]_T = 50$ mg/L (●), $[HA]_T = 100$ mg/L (▲). Open symbols correspond to ambient Cu concentrations. The lines represent 100% (dashed line) and 90% (dotted line) Cu recoveries.

To estimate the concentration of strong-binding sites in the humic acid, free Cu concentrations (i.e., the difference between $[Cu]_T$ and $[CuHA]$) were plotted as a function of total Cu concentration (Fig 5.4). Theoretical curves are also shown using previously-reported values for the conditional stability constant ($\log K_{CuHA}^{cond} \approx 12$, Table 5.1). The reported values of the constants for humic acids vary from $10^{12.0}$ to $10^{12.5}$ with

the value reported for the Suwannee River humic acid being $10^{12.0}$. The titration curve for the 50 mg/L humic acid sample lies close to that predicted for $\log K_{CuHA}^{cond} = 12$ (Fig. 5.4a). However, due to the large experimental error, the data would be as closely reproduced by any model curve with $\log K_{CuHA}^{cond} > 12$. A rough estimate of the total concentration of humic acid binding sites can be obtained from the last two experimental points in Fig. 4.4a. Assuming 1:1 stoichiometry, the estimated value is 50 nM or 1 nmol/mg of humic acids. This value is significantly lower than previously reported value of 23 nmol/mg (Xue and Sigg, 1999) and 10 nmol/mg (Kogut and Voelker, 2000). Since the Cu-humic acid complexes formed at higher total Cu concentrations were labile on the column, these experiments could not be performed under the experimental conditions used in previous studies (i.e., at higher Cu:HA ratios).

The Cu titration curve obtained with a 100 mg/L sample is not consistent with that for 50 mg/L. The measured response does not indicate saturation of the humic acid binding sites (i.e., a 1:1 increase of free Cu concentration with total Cu concentrations) (Fig. 5.4b). This behavior may be a result of the higher experimental error for the 100 mg/L sample due to the significant matrix effects at high humic acid concentrations.

Attempts were made to minimize the dissociation of Cu-HA by decreasing the elution time of the complex and hence its exposure to non-equilibrium conditions. Experiments were conducted at higher flow rates of 0.6 and 0.8 mL/min. Although the increased flow rates resulted in decreased elution times, they also lead to the decreased sensitivity of ICP-MS measurements. Since no general improvement in copper measurements was observed with the increased flow rates, all the following experiments were performed at 0.4 mL/min.

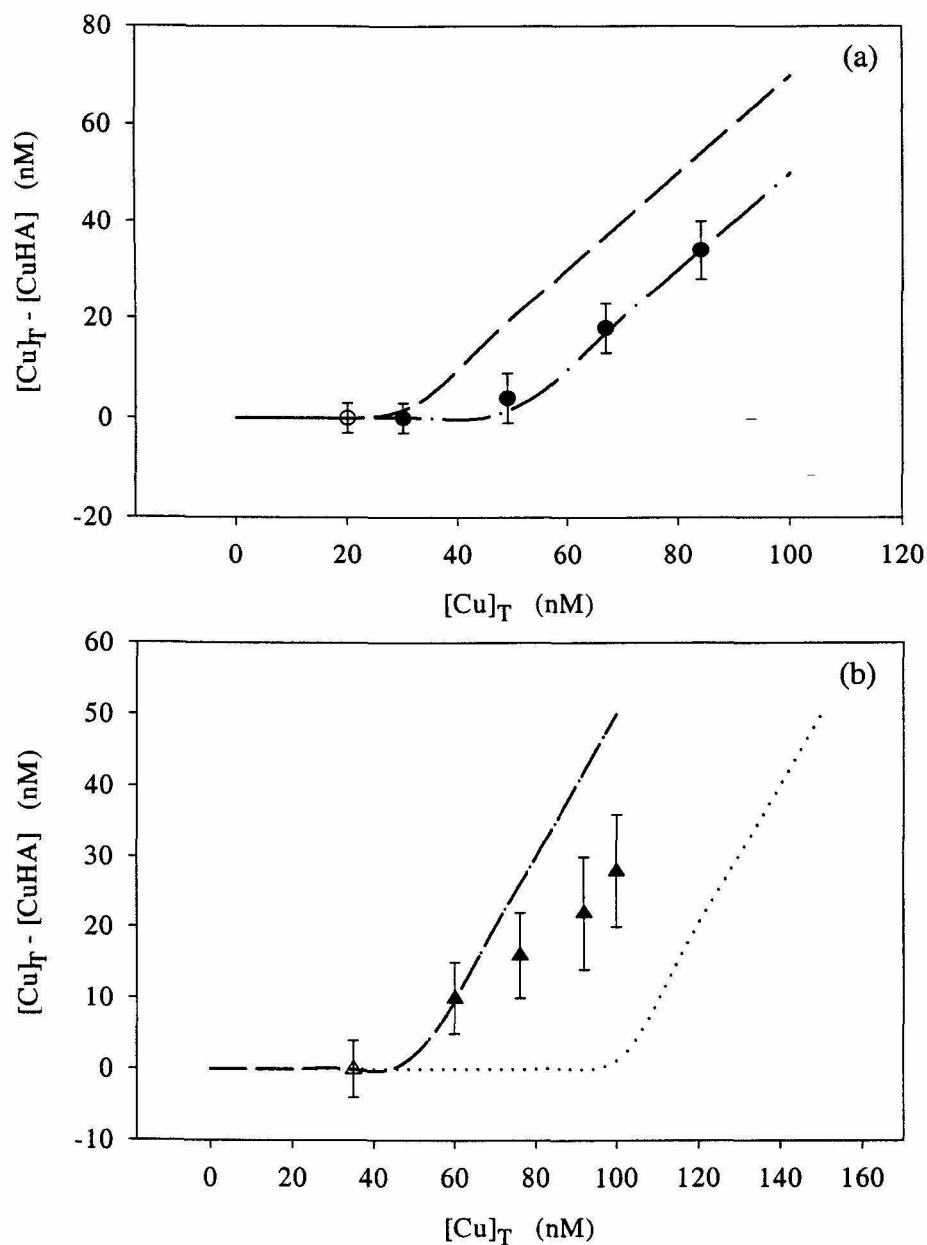


Figure 5.4. Dependence of concentration of Cu not bound with humic acids ("free" Cu) on total Cu concentration.
 Conditions: 10 mM ammonium acetate, pH = 6.
 Symbols: "free" Cu concentration calculated from the experimental data (●) $[HA]_T = 50$ mg/L (a), (▲) $[HA]_T = 100$ mg/L (b); Open symbols correspond to ambient Cu concentrations.
 Lines - "free" Cu concentration calculated with MINEQL⁺ for $\log K_{CuHA}^{cond} = 12$ and total ligand concentrations $[L]_T = 30$ nM (dashed line), 50 nM (dot-dashed line), and 100 nM (dotted line).

5.4.3 Humic acid – EDDHA competition experiments

The effect of EDDHA additions on copper speciation in humic acid samples was examined in competitive equilibration experiments. EDDHA titration experiments were conducted with 50 mg/L humic acids without any added Cu (i.e., $[Cu]_T = 2.0 \times 10^{-8}$ M) and with 100 mg/L humic acids with Cu added to a total concentration of 5.0×10^{-7} M (Fig. 5.5a and 5.6a). Copper titration experiments were performed with 100 mg/L humic acid and 1.0×10^{-5} M EDDHA (Fig. 5.5b). Each point on the graphs is the average of measurements on duplicate samples with the range shown by the error bars.

Copper recovery was greater than 97% for the titrations of 50 mg/L humic acid (no added copper) with EDDHA and in titrations of 100 mg/L humic acid and 1.0×10^{-5} M EDDHA with Cu. However, for titrations of 100 mg/L humic acid and 5.0×10^{-7} M total Cu with EDDHA, Cu recovery was greater than 90% only at high concentrations of added EDDHA (Fig. 5.6b).

The observed competition for Cu from humic acids is significantly stronger than that predicted based on the reported $\log K_{CuHA}^{cond} \approx 12$. For illustrative purposes, predicted results (for $\log K_{CuHA}^{cond} = 15$ and a total binding site concentration of 1 nmol/mg humic acid (see section 5.4.2)) are shown in Fig. 5.7 for the EDDHA titrations 50 mg/L humic acids (no added Cu) and Cu titration experiments with 100 mg/L humic acid and 1.0×10^{-5} M EDDHA.

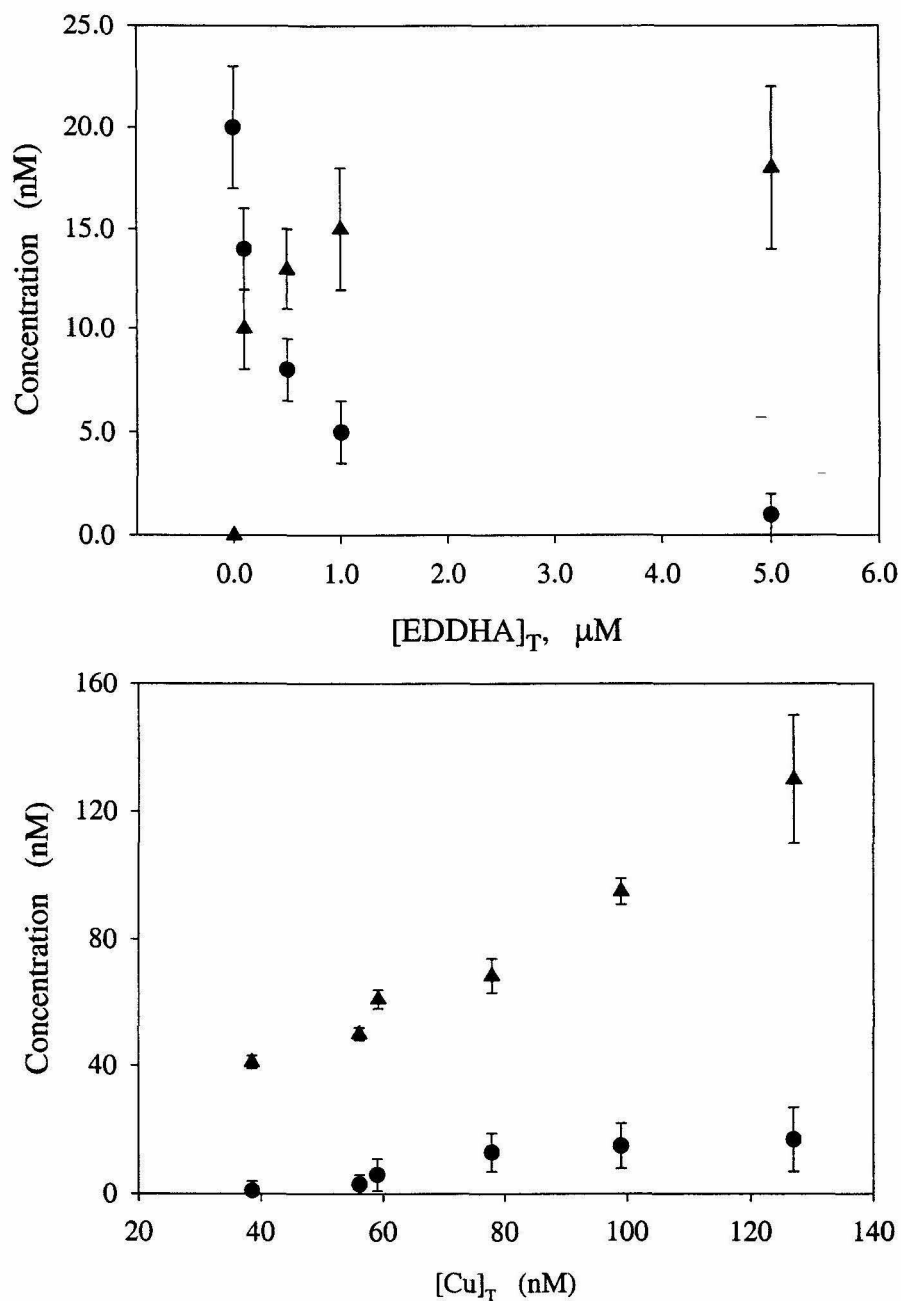


Figure 5.5. Dependence of Cu concentration in CuEDDHA and CuHA complexes as measured by ICP-MS after the SEC separation on the concentration of total EDDHA (a) or Cu (b).

Conditions: pH = 6, $I = 10$ mM;

(a) [HA] = 50 mg/L, no copper added to the sample.

Ambient $[Cu]_T = 20$ nM.

(b) [HA] = 100 mg/L, [EDDHA] = 1.0×10^{-5} M.

Symbols: (●) [CuHA] and (▲) [CuEDDHA] measured with ICP-MS.

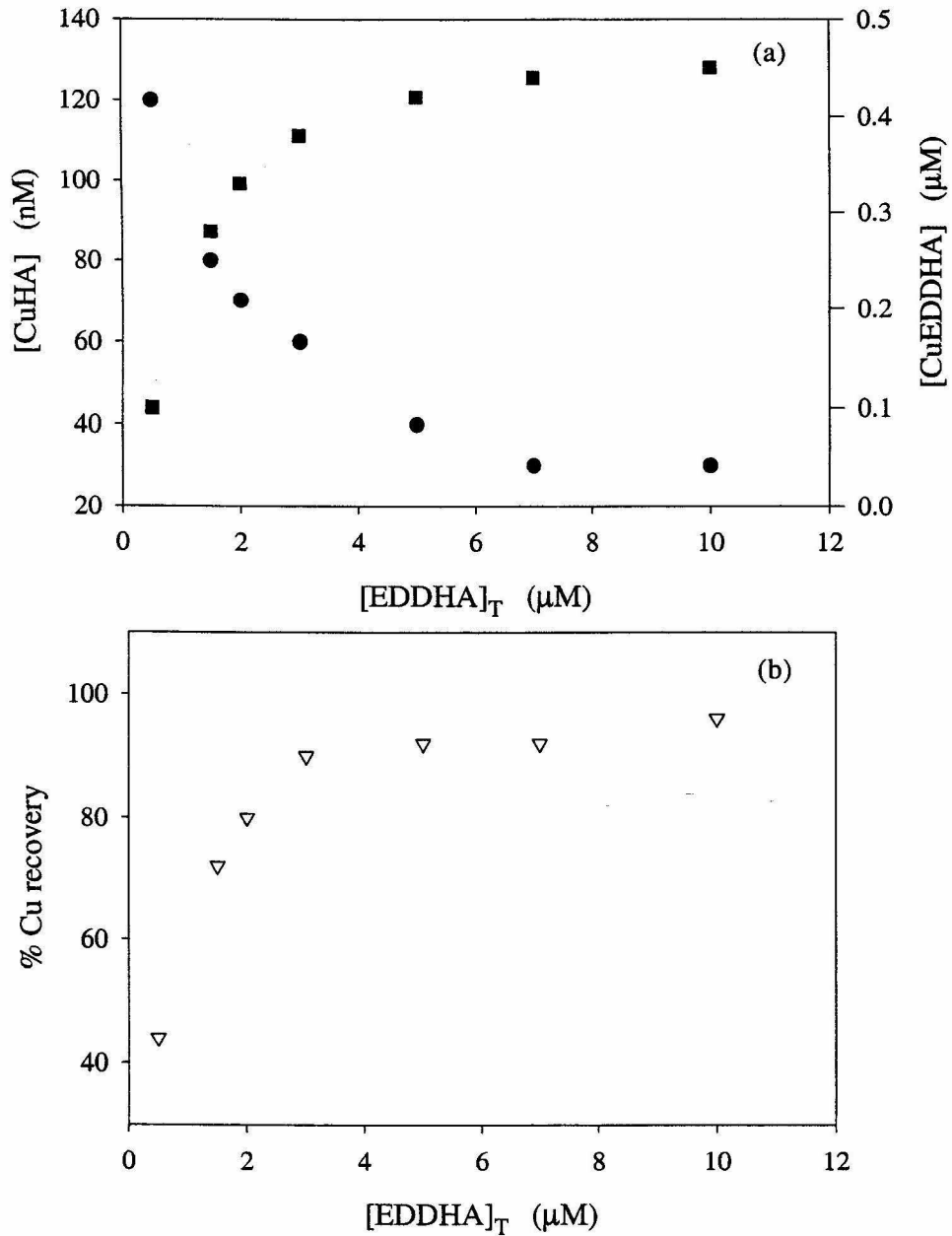


Figure 5.6. Dependence of Cu concentration in CuHA and CuEDDHA complexes as measured by ICP-MS (a) and % Cu recovery (b) on concentration of EDDHA.

Conditions: $[\text{HA}] = 100 \text{ mg/L}$, $[\text{Cu}]_T = 5.0 \times 10^{-7} \text{ M}$, 10 mM ammonium acetate, pH = 6.

Symbols: (●) $[\text{CuHA}]$, (■) $[\text{CuEDDHA}]$.

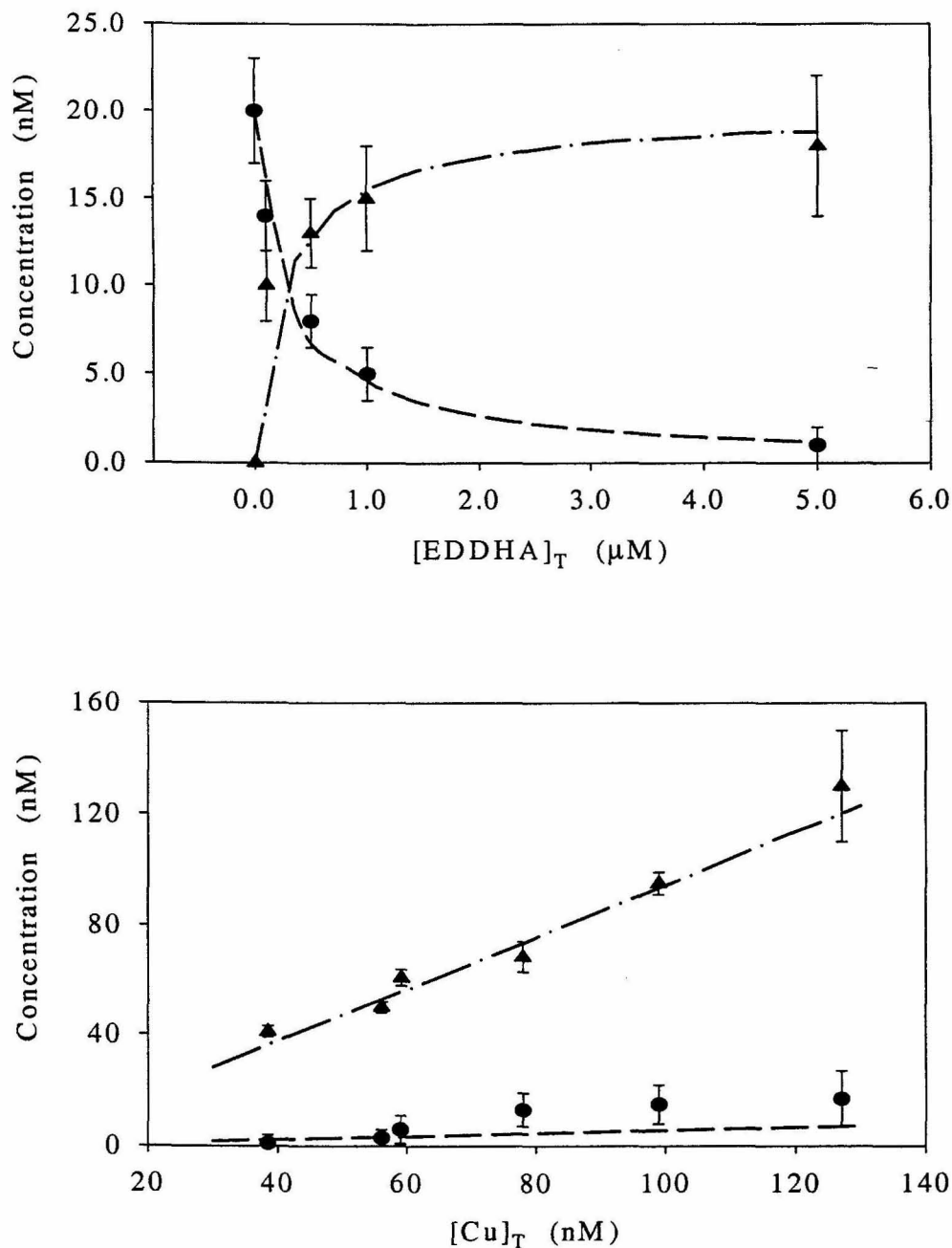


Figure 5.7. Modeling of Cu and EDDHA titrations using MINEQL⁺ for $\log K_{CuHA}^{cond} = 15$.
 Conditions: pH = 6, $I = 10$ mM;
 (a) [HA] = 50 mg/L, no copper added to the sample. Ambient [Cu]_T = 20 nM.
 (b) [HA] = 100 mg/L, [EDDHA] = 1.0×10^{-5} M.
 Symbols: (●) [CuEDDHA] and (▲) [CuHA] measured with ICP-MS;
 lines - [CuHA] (dashed) and [CuEDDHA] (dash-dotted) calculated using MINEQL⁺.

Note that, for Cu titrations of humic acid and EDDHA, EDDHA (with $\log K^{cond} = 14.1$) strongly outcompetes the humic acid for the added Cu. Conditional stability constants could not be accurately determined due to the significant experimental error associated with low Cu concentrations and high matrix effects associated with high humic acid concentrations. It must be remembered that a consistent value of the total ligand concentrations were not obtained for 50 and 100 mg/L humic acids (section 5.4.2). An accurate value for this parameter is necessary for determination of the conditional stability (see Chapter 3).

There can be significant ambiguity associated with the assignment of the total concentration of strong metal-binding sites and corresponding stability constants for mixtures of ligands such as humic acids (Voelker and Kogut, 2000). The results presented suggest that strong Cu-binding sites in humic acids are present at very low concentrations essentially comparable to the ambient Cu loading of the purified Suwannee River humic acid. Although the conditional stability constant of these strong binding sites appears to be greater than 10^{12} , these sites are not available to bind added Cu. The strong sites reported in previous studies were not detected by the SEC/ICP-MS method.

5.5 Concluding remarks

Experiments with 50 and 100 mg/L humic acids using SEC/ICP-MS method have demonstrated that Cu complexes with humic acids are kinetically labile on the column for

Cu additions comparable to ambient Cu concentrations in the humic acid samples. This suggests that strong Cu-binding sites are present in humic acids at very low concentrations or/and that ambient Cu present in the humics is complexed differently than that added.

Insufficient sensitivity of the SEC/ICP-MS method for measurements of such low Cu concentrations and the kinetic lability of Cu complexes at higher total metal concentrations prevented the accurate estimation of the conditional stability constant for Cu-humics complexes.

Chapter 6

INVESTIGATION OF COPPER-PHYTOCHELATIN COMPLEXES USING A SPECTROSCOPIC TECHNIQUE WITH BATHOCUPROINE

6.1 Introduction

Phytochelatins are small biogenic metal-binding polypeptides of the type (γ -Glu-Cys)_n-Gly with n varying from 2 to 11 (Fig. 6.1a). The structure depends on the source organism and the conditions of its exposure to metal ions (Zenk, 1996; Bae and Mehra, 1997). Cu(II) is an effective inducer (second only to Cd) of phytochelatin production in living cells (Zenk, 1996 and ref. cit.). Copper-phytochelatin complexes have been reported to be more stable than other metal-phytochelatin complexes (Leopold and Günther, 1997; Scarano and Morelli, 1997). Observed systematic variations of phytochelatin concentrations in marine particulate matter with free Cu(II) concentration suggest a copper-specific response of intracellular phytochelatin production (Ahner et al., 1997). Copper-phytochelatin complexation may influence metal toxicity in natural waters.

The information available on complexation of copper by phytochelatin is limited and often ambiguous. Questions regarding the oxidation state of copper in phytochelatin

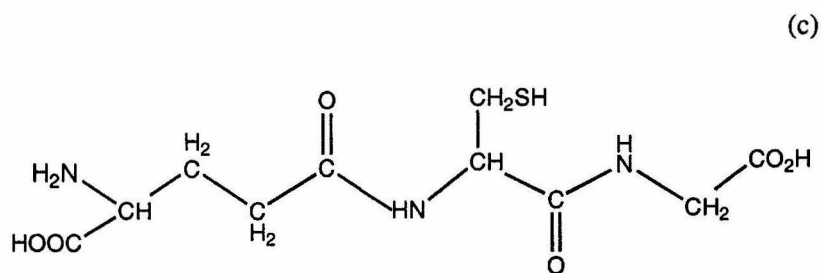
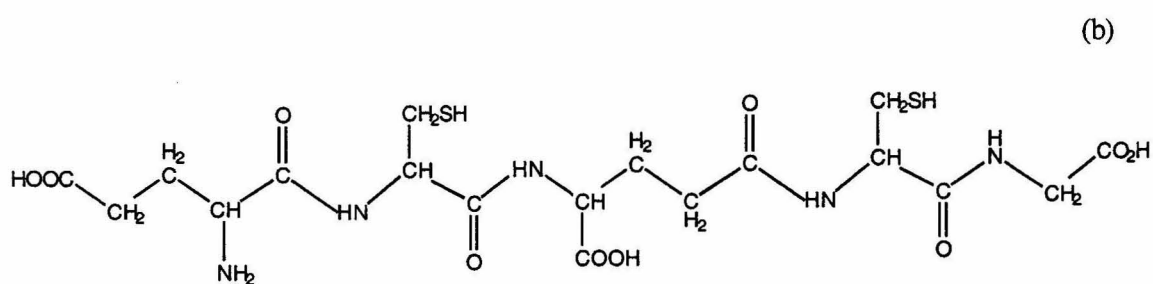
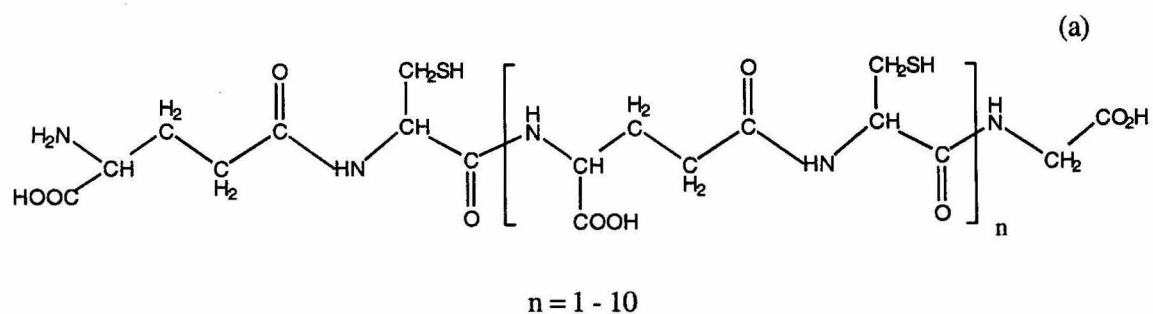


Figure 6.1. Chemical structures of phytochelatins (a), synthetic phytochelatins used in the study (b) and glutathione (c).

complexes and the stoichiometries and stabilities of these complexes remain unresolved. The goal of the present work was to investigate the stability of copper-phytochelatin complexes. Preliminary investigations indicated that Cu(II) is not stable in the presence of phytochelatin. Further studies therefore focused on the formation of Cu(I)-phytochelatin complexes which were investigated using a spectroscopic technique with bathocuproine disulfonic acid (Moffett et al., 1985). The study was performed using a synthetic phytochelatin, an oligopeptide with the structure $(\alpha\text{-Glu-Cys})_2\text{-Gly}$ (Fig. 6.1b). The synthetic phytochelatin and the native compound(s) isolated from cell cultures differ in the linkage between glutamic acid and cysteine. The synthetic peptide has been shown to have metal chelation properties similar to phytochelatin (Bae and Mehra, 1997). Thus the results of this study should be applicable to the native copper-phytochelatin complexes.

6.2 Background

The properties of phytochelatin complexes with various metals have been examined for both native complexes and those reconstituted by addition of the metal to isolated or synthesized phytochelatin. These studies are summarized in Table 6.1. Phytochelatin contains several functional groups that can be important in metal binding including thiol, amino and carboxylic groups.

Table 6.1. Summary of previous studies of metal-phytochelatin studies

phytochelatin (P) source or type	metals	summary of experimental work	reference
yeasts (<i>Schizosaccharomyces pombe</i> , <i>Candida glabrata</i>) synthetic (Glu-Cys) ₂ Gly	Cd, Hg(II), Pb	<u>Isolation/synthesis</u> : P's isolated by combination of ion-exchange, gel filtration and RP-HPLC; synthetic peptide obtained commercially. <u>Analytical methods</u> : UV-vis for metal-P complexes; separation of Hg(II)-P complexes and apo-P's by RP-HPLC; electrospray-mass spectrometry for Hg(II)-P complexes. <u>Studies performed</u> : (1) effect of metals on UV-vis spectra of P's; (2) exchange of Hg(II), Cd(II), and Pb(II) between G and P's; (3) exchange of Hg(II) between apo-P's (isolated and synthetic)	Bae and Mehra (1997)
yeasts (<i>Schizosaccharomyces pombe</i>)	Cu(I), Cu(II), Zn, Cd	<u>Isolation/synthesis</u> : P's isolated by combination of gel filtration and anion exchange and concentrated by ultra-filtration. <u>Analytical methods</u> : atomic absorption for metals in P complexes; UV-vis for metal-P complexes; Ellman's method for sulfhydryl groups; amino acid analyses of P. <u>Studies performed</u> : (1) determination of metals, sulfhydryl groups and acid labile sulfides in metal-P complexes; (2) UV spectra of different subspecies of Cd-P complexes and effect of the salt concentration on the spectra; (3) induction of P by Cu, Zn and oligosaccharides; (4) effect of Cu valence state on the UV spectra of Cu-P complexes; (5) determination of chemical structure of P.	Hayashi et al., (1991)
plants (<i>Rauvolfia serpentina</i> ; <i>Silene cucubalus</i>)	Cd	<u>Isolation/synthesis</u> : P's isolated by gel filtration. <u>Analytical methods</u> : Ellman's method for sulfhydryl groups; atomic absorption for Cd, colorimetric S ²⁻ -test with methylene blue method. <u>Studies performed</u> : kinetic measurements of P and sulphide content in Cd-treated suspension of the cultures.	Kneer and Zenk, (1996)

Table 6.1 (continued). Summary of previous studies of metal-phytochelatin studies

phytochelatin (P) source or type	metals	summary of experimental work	reference
plant (<i>Silene vulgaris</i>)	Cu(II), Zn, Cd, Pb	<u>Isolation/synthesis</u> : P's isolated by gel filtration and RP-HPLC. <u>Analytical methods</u> : ICP-MS for metal analysis; UV-vis for metal-P complexes; electrospray-mass spectrometry for Cu and Cd-P complexes. <u>Studies performed</u> : (1) <i>in-vitro</i> heavy metal saturation assay and <i>in-vivo</i> stress experiments; (2) Cu and Cd competition experiments for P.	Leopold and Günther, (1997)
plants (<i>Silene vulgaris</i>) and tomato	Cu(II), Zn, Cd, Pb	<u>Isolation/synthesis</u> : P's isolated by gel filtration. <u>Analytical methods</u> : ICP-MS for metal analysis; UV-vis for metal-P complexes; electrospray-mass spectrometry for Cu and Cd-P complexes. <u>Studies performed</u> : (1) evaluation of coupled SEC/ICP-MS technique for metal-P binding; (2) evaluation of electrospray for metal-P complexes characterization	Leopold et al., (1998)
yeasts (<i>Schizosaccharomyces pombe</i> , <i>Candida glabrata</i>)	Cu(I)	<u>Isolation/synthesis</u> : P's isolated from Cd-P complexes by RP-HPLC at pH 2. <u>Analytical methods</u> : atomic adsorption for Cu in P; fractionation of Cu(I)-P complexes by gel filtration; UV-vis and luminescence for Cu(I)-P complexes; total glutathione analyses by a cyclic assay. <u>Studies performed</u> : effect of Cu(I) on UV-vis and luminescence spectra of P's and thionein obtained by Cu(I) exchange between P/thionein and CuCl or Cu(I)-G.	Mehra and Mulchandani, (1995)
yeasts (<i>Schizosaccharomyces pombe</i>)	Cd, Cu(II)	<u>Isolation/synthesis</u> : P's isolated by combination of ion-exchange, gel filtration and RP-HPLC. <u>Analytical methods</u> : atomic absorption for metals in P complexes; luminescence; gel filtration; amino acid analyses of P; analyses of labile sulfur with ³⁵ S; UV-vis.	Reese et al., (1988)

Table 6.1 (continued). Summary of previous studies of metal-phytochelatin studies

phytochelatin (P) source or type	metals	summary of experimental work	reference
yeasts (<i>Schizosaccharomyces pombe</i>)	Cd, Cu(II)	<u>Studies performed:</u> (1) induction of P by Cd, Cu and Zn; (2) determination of chemical structure of Cd and Cu(II) induced P; (3) determination of metal-binding stoichiometries; (4) effects of pH on stability of metal-P complexes; (5) effects of ionic strength, presence or absence of oxygen, pH on luminescence of Cu-P complexes.	Reese et al., (1988)
marine diatom (<i>phaeodactylum tricornutum</i>)	Cu(II), Pb, Cd, Zn	<u>Isolation/synthesis:</u> P's isolated by gel filtration. <u>Analytical methods:</u> differential pulse polarography at dropping mercury electrode; atomic adsorption for metals in P. <u>Studies performed:</u> (1) effect of pH on polarographic behavior of metal-P complexes; (2) polarographic behavior of Cd-P and Pb-P complexes reconstructed from their depleted forms (3) comparison of polarographic behaviors of metal complexes of P and metallothioneins.	Scarano and Morelli, (1997)

6.2.1 Chelating functional groups

Glutathione, γ -Glu-Cys-Gly (Fig. 6.1c), is a precursor to the biosynthesis of phytochelatin. Similarities in metal-binding by glutathione (G) and phytochelatin may be expected. NMR and EXAFS studies have indicated that glutathione binds Cu(I) only through the thiol group over a range of pH values and Cu(I):G molar ratios (Corazza et al., 1996). With oxidized glutathione (in which two G molecules are dimerized through a disulfide bond), binding of Cu(II) occurs through nitrogen- and oxygen-containing functional groups (Piu et al., 1995). In general, thiol-containing ligands are unstable in the

presence of Cu(II). It has been suggested that Cu(II) catalyzes oxidation of the thiol sulfur and formation of a disulfide bridge (Pecci et al., 1997).

6.2.2 Oxidation state of copper in phytochelatin complexes

The issue of the oxidation state of copper has not been fully resolved in studies of phytochelatin. Cu(II) has been shown, in both laboratory and field studies, to be an effective inducer of phytochelatin production in plants and fungi (Zenk, 1996 and ref. cit.). Cu(II) additions to phytochelatin isolated from the plant *Silene vulgaris* were observed to result in the formation of a Cu-phytochelatin complex that could be isolated by SEC and quantified by ICP-MS (Leopold and Günter, 1997). However, we observed substantial loss of copper in similar SEC/ICP-MS experiments with synthetic phytochelatin solutions amended with Cu(II) (Appendix 6A). These observations suggest that the Cu(II)-phytochelatin complex is labile under chromatographic conditions (possibly due to redox reaction between Cu(II) and the thiol groups of phytochelatin).

Several analytical studies have indicated that copper in phytochelatin complexes occurs in the +I oxidation state. UV spectra of the copper-phytochelatin complex isolated from cultures of the yeast *Schizosaccharomyces pombe* correspond to phytochelatin substituted with Cu(I) rather than Cu(II) (Hayashi et al., 1991). This copper phytochelatin complex exhibits luminescence with an emission peak at 619 nm when excited in the UV (at 290 nm) (Reese et al., 1988). Similar luminescence has been reported for Cu(I) complexes with small thiolate ligands, including cysteine, glutathione, and 2-mercaptoethanol

sulfonate (Winge, 1991 and ref. cit.). Such luminescence is observed only for Cu(I) ions since the paramagnetic Cu(II) ion quenches emission.

A potential complication in ascertaining the oxidation state of copper in phytochelatin complexes arises from the addition of reducing agents such as 2-mercaptoethanol or dithiothreitol. This practice is intended to avoid phytochelatin oxidation during its isolation from cell cultures and in experiments on metal complexation. Small thiol-containing ligands, such as mercaptosuccinic acid, mercaptoethanol, and cysteine, have been reported to be oxidized in the presence of Cu(II), which undergoes reduction to Cu(I) (Klotz et al., 1958; Klothoff and Stricks, 1951; Pecci et al., 1997). Therefore, it is unlikely that a Cu(II) complex with phytochelatin could survive under such conditions.

6.2.3 Stability of metal-phytochelatin complexes

Limited information is available on the stability of phytochelatin complexes with various metals; most studies have reported only the relative stability of the complexes. A study using a coupled SEC-ICP/MS technique has shown that even a ten-fold Cd excess did not displace Cu from its phytochelatin complex while Cd was displaced by equimolar amounts of Cu(II) (Leopold and Günter, 1997). In polarographic studies, Cu, added as Cu(II), was found to form more stable complexes with phytochelatin than Cd or Pb. Both Cd and Pb were lost from their complexes at pH less than 4 and less than 3 respectively while Cu was not completely displaced from its complex with phytochelatin even at pH less than 2 (Scarano and Morelli, 1997).

Depending on the experimental conditions polarographic studies can either overestimate or underestimate stability of metal complexes with thiol-containing ligands. It has been shown for Cu-glutathione complex that the complex may be stabilized by the adsorption on the electrode or that complex dissociation can occur in the diffusion layer surrounding the electrode (Leal and van den Berg, 1997). Complex dissociation in the electrode diffusion layer was probably responsible for the lack of detection of Zn-phytochelatin complex by polarographic measurements (Scarano and Morelli, 1997).

6.2.4 Stoichiometries of Cu-phytochelatin complexes

Interpretations of metal complexation by phytochelatins has been often based on an analogy with metallothioneins, heavy metal-complexing cysteine-rich polypeptide (MW 6800 Da) produced in mammalian systems. X-ray crystallographic and NMR spectroscopic investigations have revealed two metal clusters in metallothioneins (Otvos and Armitage, 1980; Robbins et al., 1991; Wüthrich, 1991). The metal is bound through thiol groups of cysteines within these clusters. Each cluster binds 6 monovalent metal ions such as Cu(I). Consistent with this interpretation, luminescence spectra obtained by titrating metallothionein with Cu(I) demonstrate increased luminescence with the addition of up to 6 mol equivalents of Cu(I) and an even more substantial increase upon addition of 6 more equivalents. At higher molar ratios, luminescence decreased. This has been attributed to destabilization of the clusters (Mehra and Mulchandani, 1995).

Complexes of phytochelatin with Cu(I) exhibit luminescence with similar quantum yield (Reese et al., 1987). By analogy with metallothioneins, the effects of Cu(I) on

luminescence spectra have been interpreted in terms of the formation of polynuclear complexes (complexes containing multiple metal centers) (Mehra and Mulchandani, 1995). In this study, luminescence spectra were recorded for 50 μM phytochelatin with $n = 2$ and 3 at Cu(I)/P molar ratios ranging from 0.5 to 4. Luminescence increased linearly with addition of 0.5 - 1.0 mol equivalents of Cu(I) for $n = 2$ or 0.5 - 1.5 mol equivalents for $n = 3$; addition of the next 0.25 mol equivalents for $n = 2$ or 0.5 mol equivalents for $n = 3$ caused an even stronger increase in luminescence. In both cases the luminescence decreased after more Cu(I) was added. Based on these observations, Cu(I):P stoichiometric ratios of 1.25 ($n = 2$) and 2.0 ($n = 3$) were assigned and polynuclear structures proposed for these complexes (Mehra and Mulchandani, 1995).

Although formation of Cu-phytochelatin polynuclear clusters is possible, there are important differences between the phytochelatin and metallothionein systems. A metal cluster formed with phytochelatin would be an oligomer of multiple peptides, whereas the clusters formed in metallothionein are enfolded by a single polypeptide chain. Thus the justification of the analogy between phytochelatin and metallothionein is not clear.

However, polynuclear complexes have been reported for complexes of Cu(I) with some small thiol-containing ligands. Complexation of Cu(I) by penicillamine, which is structurally similar to glutathione, has been interpreted in terms of formation of a $\text{Cu}_5\text{L}_4^{3-}$ complex ($\text{L} = \text{penicillamine}$). The results of comparable Cu(I) titrations of glutathione could be explained with only the formation of 1:1 and 1:2 Cu(I)-glutathione complexes (Österberg et al., 1979). In contrast, EXAFS and NMR studies of copper-glutathione complexes showed that Cu(I) is coordinated to three sulfur atoms and a stoichiometry of $\text{CuG}_{1,2}$ with bridging S atoms in a polynuclear complex (Corazza et al., 1996). This

study was performed at lower ionic strength (0.1 M vs. 0.5 M) and higher concentration range ($[\text{Cu}]_{\text{T}} = 50$ and 100 mM vs. $[\text{Cu}]_{\text{T}} = 0.04 - 0.6$ mM; $[\text{G}]_{\text{T}} = 100$ mM vs. $[\text{G}]_{\text{T}} = 2 - 40$ mM) than electrochemical titrations of glutathione (Österberg et al., 1979), which may account for the formation of different species. Since the lower concentration range is used in the present study, only 1:1 and 1:2 Cu:G complexes are considered here (see section 6.5.2). Formation of polynuclear complexes in Cu(I)-cysteine-glutathione systems has also been reported (Tran-Ho et al., 1997).

A 1:2 stoichiometry has been suggested for the complex of Cu with phytochelatin ($n = 2$) based on electrospray studies (Leopold and Günter, 1997). However, in another using electrospray mass spectrometry, neither Cu or Cd complexes with phytochelatin could be identified (Leopold et al., 1998). The absence of expected signals at m/z of 1140 (for 1:2 Cu-phytochelatin) and 1188 (for 1:2 Cd-phytochelatin) is probably due to oxidation in the electrospray interface. The observed signal at m/z of 538 can be attributed to the oxidized form of phytochelatin.

The interpretation of experimental results is complicated by the susceptibility of phytochelatin to redox transformations and the limitations of the analytical techniques applied. These various (and sometimes conflicting) observations indicate that complexation of metals (specifically copper) with phytochelatin is not well understood.

6.4 Experimental methods

6.4.1 Reagents

A Cu(I) stock solution of 2.5 mM was prepared by dissolving cuprous chloride (Mallinckrodt Baker) into a solution of 0.1 M hydrochloric acid and 0.7 M sodium chloride (EM Science). Bathocuproine-3,3'-disulfonic acid disodium salt (bathocuproine, Sigma, minimum 98%), glutathione (Sigma, minimum 98%) and phytochelatin were dissolved in 0.05 M phosphate buffer (KH_2PO_4 from Mallinckrodt Baker and NaOH from TitriStar) at pH 6.5 to obtain stock solutions with the final concentration of 2.0 mM. Solutions were freshly prepared prior to each experiment using 18 Ω cm water (MilliPore) deoxygenated with high-purity argon under vacuum. Synthetic phytochelatin, (α -Glu-Cys)₂Gly (Fig. 6.1b), was used in all experiments (Caltech Biopolymer Synthesis Center). Note that the synthetic phytochelatin is referred to as phytochelatin in sections 6.4 - 6.6. To insure that all the thiol groups were fully reduced, the phytochelatin (20 mM) was equilibrated for 9 days in a solution of 0.1 M sodium borohydride (Sigma) acidified to pH 6.5 with hydrochloric acid (Slinski, 1999). The exact concentration of the reduced phytochelatin was determined by the method of thiol substitution with nitrous acid calibrated with reduced glutathione (Jocelyn, 1987). Solutions were prepared in a nitrogen-filled glove box.

6.4.2 Analytical methods

Absorbance was measured with a Hewlett Packard spectrophotometer (HP 8453 UV Vis); full spectra (200-800 nm) were acquired and measurements at specific wavelength for individual samples were normalized to a background signal outside the absorption band for the target analyte. The spectral characteristics for the Cu(I)/bathocuproine complex are $\lambda_{\max} = 480$ nm, $\epsilon_{\max} = 12,800$ L mol⁻¹ cm⁻¹, in good agreement with previously reported values, $\lambda_{\max} = 483$ nm, $\epsilon_{\max} = 12,250$ L mol⁻¹ cm⁻¹ (Blair and Diehl, 1961).

Low-temperature X-band EPR spectra were recorded at 9.4 GHz (X-band) using a Bruker EMX EPR spectrometer, with modulation setting of 8.0 G. The microwave power varied for samples with different [Cu]_T and was 6.4 mW for [Cu]_T = 0.5 mM, 0.6 mW for [Cu]_T = 1.0 mM and 10.1 mW for [Cu]_T = 2.1 mM.

6.4.3 Titration experiments

Titration experiments were performed in a 1-cm cuvette with solution absorbance being measured at each titration point. In each type of titration experiment, the first two reagents were added into the cell by a one-time addition and mixed by shaking. The initial volume of the solution was 2 mL. The titrant was added to the cuvette in 10 μ L aliquots and the solution was mixed by shaking after each addition. The total volume

change was not more than 12% in all experiments; appropriate dilution factors were included for data fitting (see section 6.4.4).

Preliminary experiments were conducted to determine the appropriate equilibration time. Absorbance was measured as a function of time for a Cu(I)/bathocuproine/glutathione system at pH 6.5 in 0.05 M phosphate buffer in two types of experiments. For Cu(I) titration experiments, Cu(I) was added to a mixture of 1.0×10^{-4} M bathocuproine and 2.0×10^{-4} M glutathione in 10 μ L increments at 10 minute intervals (the solution was mixed after each addition) up to the final concentration of 0.48×10^{-4} M. Then the absorbance of the solution was measured every 10 minutes for an hour. Similarly, for bathocuproine titration experiments, bathocuproine was added in 10 μ L increments up to the final concentration of 1.7×10^{-4} M to the mixture of 4.0×10^{-4} M glutathione and 0.84×10^{-4} M Cu(I). After the last addition, the absorbance of the solution was measured every 10 minutes for an hour. A constant level of absorbance was achieved in less than 10 minutes after the final addition of reagents in both Cu(I) and bathocuproine titrations. The change of absorbance in the final solution was less than $\pm 5\%$ within an hour. Based on the results of these experiments, a 10-minute pre-equilibration time was used in all titration experiments.

6.4.4 Experimental data modeling

MINEQL⁺ (Schecher and McAvoy, 1998) was used for initial modeling to determine the type of complexes needed to fit the observations and to evaluate the consistency

of experimental and literature data. The constants were optimized later using FITEQL 4.0 (Herbelin and Westall, 1999).

Conditional stability constants (as defined in Table 6.2) for the experimental pH 6.5 and ionic strength 0.05 M were used in both programs, so the protonation constants and the ionic strength corrections were not included.

Table 6.2. Symbols	
Symbol	Significance
G	glutathione, γ -Glu-Cys-Gly
P	phytochelatin (γ -Glu-Cys) _n -Gly; in this study: synthetic phytochelatin, (α -Glu-Cys) ₂ -Gly
B	bathocuproine-3,3'-disulfonic acid
K_{HL}	protonation constant for ligand L, where $K_{HL} = \frac{[HL]}{[H^+][L]}$; charges omitted, concentration in mole/liter.
β_{ML_x}	overall stability constant for a complex ML_x , where $\beta_{ML_x} = \frac{[ML_x]}{[M][L]^x}$; charges omitted, concentrations in mole/liter.
$\beta_{CuL_x}^{cond}$	conditional stability constant for a complex ML_x , where $\beta_{ML_x} = \frac{[ML_x]}{[M][L']^x}$ and L' includes both free and protonated forms of the ligand L; $[ML_x]$ includes protonated complexes; charges omitted, concentrations in moles/liter; expressed in terms of proton activity and concentrations of all other species.

The constants are expressed in terms of activity for proton and in terms of concentrations for all other species. The effect of the side reactions for Cu(I) was negligible.

Dilution was not taken into account in initial calculations performed using MINEQL⁺ but a dilution factor was included in FITEQL 4.0 optimization of the conditional stability constants. The uncertainty introduced into the values of conditional stability constants due to the change in the ionic strength during titrations was within the experimental error. A 100% purity of reagents was assumed in all calculations.

For the optimization of the constants using FITEQL 4.0, estimated or literature values of conditional stability constants (when available) were set as fixed parameters and the remaining constants were optimized. If the fit of the experimental data could be improved, other constants were also included into the optimization procedure. However, simultaneous optimization of all the conditional stability constants of interest could not be performed in most cases. Sensitivity of the optimization procedure to the stability constants of complexes present at low concentrations (less than 5% $[\text{Cu}]_{\text{T}}$) was low. For that reason, stability constants for such species were generally set as fixed values.

To improve the sensitivity of the optimization procedure, wherever possible, each complex conditional stability constant optimization was performed using the experimental data (i.e., the part of the titration curve) where the complex was expected to be predominant.

6.4.5 Estimation of Cu(I)-bathocuproine (CuB_2) conditional stability constant

Stability constants for Cu(I) complexes have been reported for a few phenanthroline derivatives but not for bathocuproine (Table 6.3, Fig. 6.2) (Martell et al., 1993).

Table 6.3. Protonation and stability constant of Cu(I) complexes with phenanthroline and its derivatives ($I = 0.5$) (Martell et al., 1993)		
Ligand	Log K_{HL}	log β_{CuL_2}
phenanthroline	5.03	15.82
2-methyl-1,10-phenanthroline	5.3	16.95
2,9-dimethyl-1,10-phenanthroline	5.88	19.1
bathocuproine	5.8	[18.8]*

* - calculated from LFER, see text

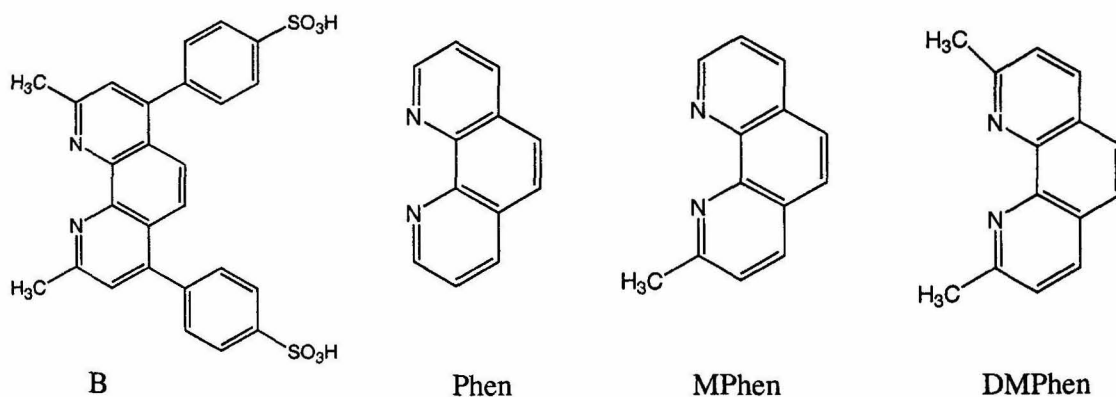


Figure 6.2. Chemical structures of bathocuproine (B), phenanthroline (Phen) and of the derivatives used in linear free energy relations estimations of copper(I)-bathocuproine stability constant: 2-methyl-1,10-phenanthroline (MPhen), 2,9-dimethyl-1,10-phenanthroline (DMPhen).

Comparable affinities for Cu(I) may be expected for bathocuproine and the similarly substituted compound 2,9-dimethyl-1,10-phenanthroline (neocuproine).

For imidazole and its derivatives in aqueous solution, a linear free energy relationship (LFER) has been established between complex stability and ligand basicity for sterically unhindered ligands (Kapinos et al., 1998). Analogously, trends consistent with the effects of electron-donating or withdrawing substituents on basicity of the phenanthroline nitrogen have been found for the 1:1, 1:2 and 1:3 metal-phenanthroline complexes (Piu et al., 1997).

Figure 6.3 shows an LFER for Cu(I) complexation by phenanthroline derivatives. The overall stability constant for the Cu(I) complex ($I = 0.5$) is related to the protonation constant by the expression $\log \beta_{ML_2} = 3.8 [\log K_{HL}] - 3.4$. If it is assumed that bathocuproine forms a planar copper(I) complex with no additional steric constraints, a stability constant for the Cu(I)-bathocuproine complex (CuB_2) of $\log \beta_{ML_2} = 18.8$ ($I = 0.5$) can be estimated from the $\log K_{HL} = 5.8$ (Blair and Diehl, 1961). The corresponding conditional stability constant for pH 6.5 and ionic strength 0.05 M (calculated using Davies equation) is $\log \beta_{ML_2}^{cond} = 18.5$.

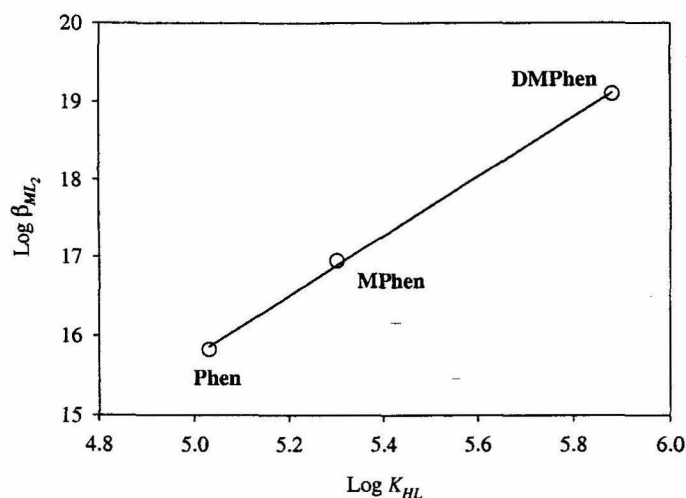


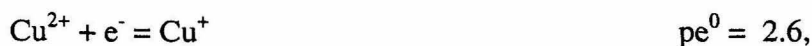
Figure 6.3. Relationship between $\text{Log } \beta_{ML_2}$ and $\text{Log } K_{HL}$ for copper(I) and 1:2 complexes of phenanthroline (Phen), 2-methyl-1,10-phenanthroline (MPhen), 2,9-dimethyl-1,10-phenanthroline (DMPhen). The least-square line is drawn through the indicated three points. All plotted equilibrium constants refer to aqueous solutions at 25 °C and $I = 0.5$ M.

6.5 Results and discussion

6.5.1 Valence state of copper ions bound to phytochelatin

The thermodynamic stability of Cu(II) in the presence of phytochelatin can be determined based on the standard potential of the corresponding redox reaction. The standard reduction potential of Cu(II)/Cu(I) couple is known (for 25 °C) (Weast, 1976). The standard reduction potential of disulfide/thiol couple in phytochelatin has not been reported, but an estimate can be made using the reported standard reduction potential of

cystine/cysteine couple (Schwarzenbach et al., 1993). The reported values for the half reactions are:



The overall redox reaction is thus energetically favorable at standard conditions, $\Delta G^0 = -17.2$ kJ/mole. For the experimental pH of 6.5, reaction is even more favorable, $\Delta G_{\text{pH}=6.5}^0 = -54.3$ kJ/mole. It is reasonable to expect that reaction of Cu(II) reduction by the thiol groups of phytochelatin is also thermodynamically favorable.

However, it is possible that a Cu(II)-phytochelatin complex might persist as a metastable species. The oxidation state of copper in the presence of phytochelatin was assessed spectroscopically using bathocuproine, a highly specific Cu(I)-binding reagent, and by EPR.

6.5.1.1 Bathocuproine assay

Bathocuproine forms a strongly-colored 1:2 Cu(I) complex (CuB_2) with an absorbance maximum at 480 nm (Blair and Diehl, 1961); absorbance at this wavelength is negligible in the presence of Cu(II). It may be expected that, if Cu(II) is reduced to Cu(I) by phytochelatin in the presence of bathocuproine, the colored CuB_2 will be formed.

The absorbances of solutions containing (i) bathocuproine alone, (ii) bathocuproine and copper added as Cu(II), and (iii) bathocuproine, copper added as Cu(II) and phytochelatin were measured at 480 nm. As seen in Fig. 6.4, formation of the colored Cu(I)-bathocuproine complex is observed upon addition of phytochelatin. The results are con-

sistent with reduction of Cu(II) to Cu(I) by phytochelatins followed by complexation of Cu(I) by bathocuproine. The signal of the Cu(I)-bathocuproine complex was smaller at the higher concentration of the added phytochelatins suggesting competition between phytochelatins and bathocuproine for Cu(I).

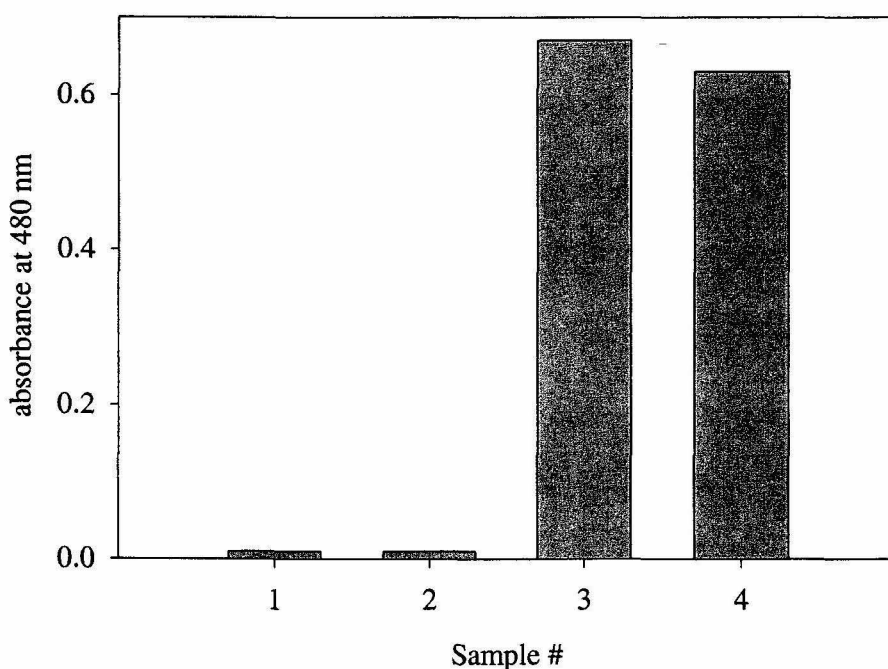


Figure 6.4. Absorbance at 480 nm due to formation of 1:2 Cu(I)-bathocuproine complex in the presence of phytochelatins; copper added as Cu(II).
Conditions: pH = 6.5, $I = 0.05$ mM (phosphate buffer);
Sample # 1: 0.2 mM bathocuproine;
Sample # 2: 0.2 mM bathocuproine and 3 μ M Cu(II);
Sample # 3: 0.2 mM bathocuproine, 3 μ M Cu(II) and 0.14 mM phytochelatins;
Sample # 4: 0.2 mM bathocuproine, 3 μ M Cu(II) and 0.28 mM phytochelatins.

6.5.1.2 EPR analysis

The instability of Cu(II) in the presence of phytochelatin, indicated by the bathocuproine assay, was confirmed by EPR analysis. A copper EPR signal is characteristic of the unpaired electron in the d^9 state of Cu(II). Complexes of Cu(I) in a configuration in which the unpaired electrons are spin-coupled are silent in the EPR spectrum. Samples were prepared for EPR by adding Cu(II) in varying ratios to phytochelatin and immediately freezing the solutions in liquid nitrogen. As seen in Fig. 6.5, the EPR signal of Cu(II) is observed only when copper is in excess of phytochelatin.

The results of these experiments indicate that Cu(II) is reduced in the presence of phytochelatin. Since Cu(I) appears to be the stable form of copper in the presence of phytochelatin, formation of the Cu(I)-phytochelatin complex was investigated using a spectroscopic technique in which bathocuproine is added as a competing ligand and the formation of the 1:2 copper-bathocuproine complex is followed spectrophotometrically (Moffett et al., 1985).

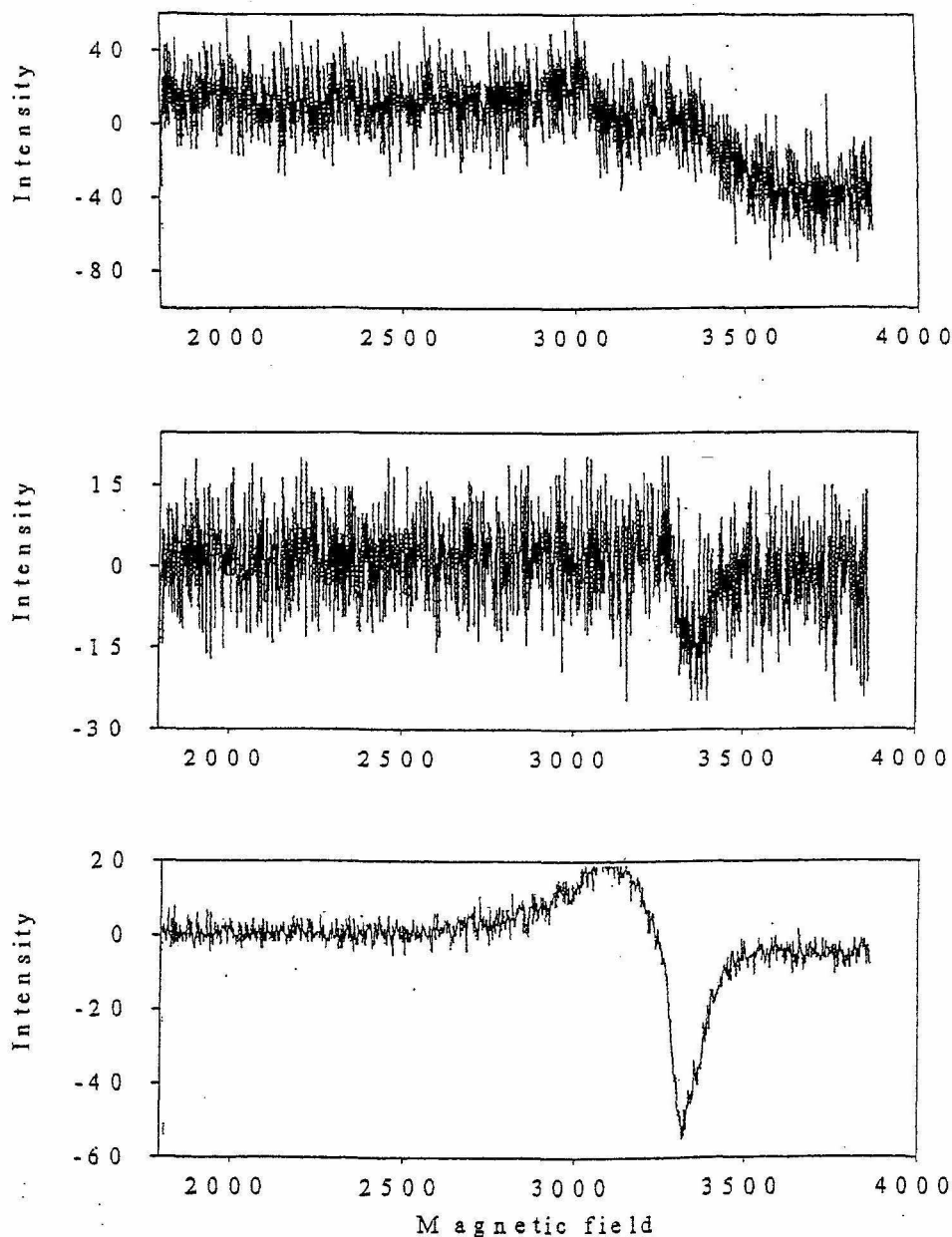


Figure 6.5. EPR spectra of frozen solutions of copper(II) added to 1.7 mM phytochelatin (P) in 50 mM phosphate buffer, pH 6.5. Ratios of Cu(II) to phytochelatin (Cu:P) and total copper concentrations $[Cu]_T$ were: (a) Cu:P = 0.3, $[Cu]_T$ = 0.5 mM; (b) Cu:P = 0.625, $[Cu]_T$ = 1.0 mM; (c) Cu:P = 1.25, $[Cu]_T$ = 2.1 mM; (Note that, in sample (c), precipitation of Cu(II) hydroxide was observed visually). Instrumental parameters: microwave power (a) 6.4 mW, (b) 0.6 mW, (c) 10.1 mW.

6.5.2 Investigation of Cu(I)-glutathione complexation using a spectroscopic technique with bathocuproine

A validation study for the bathocuproine spectroscopic method was performed using glutathione, which forms Cu(I) complexes of known stability (Table 6.4) (Österberg et al., 1979, see also section 6.2.4). Conditional stability constants for copper-glutathione complexes, corrected using the Davies equation for an ionic strength of 0.05 M and pH 6.5, are given in Table 6.5. The conditional stability constant for the complex CuB_2 ($\log \beta_{\text{CuB}_2}^{\text{cond}} = 18.5$) was estimated from a LFER (see section 6.4.5). These reported and estimated constants were used to validate the spectroscopic method by comparison of observed and predicted equilibrium Cu(I) speciation.

Table 6.4. Reported values of Cu(I)-glutathione (G) stability constants ($\log \beta_{\text{CuL}_x}$ and $\log \beta_{\text{HL}_x}$, $I = 0.5$) (Österberg et al., 1979)

Complex	$\log \beta_{\text{CuL}_x}^{\text{cond}}$	Complex	$\log \beta_{\text{HL}_x}$
CuHG^-	24.9	HG	9.37
		H_2G	17.99
$\text{Cu}(\text{HG})_2^{3-}$	38.8	H_3G	21.47
		H_4G	23.81

Table 6.5. Conditional stability constants (pH = 6.5, $I = 0.05$) of Cu(I) - glutathione (G) complexes ($\log \beta_{\text{CuL}_x}^{\text{cond}}$) calculated from the reported values with Davies equation

Complex*	$\log \beta_{\text{CuL}_x}^{\text{cond}}$
CuG	13.4
CuG ₂	15.3
* - "CuG and CuG ₂ " are the same complexes as "CuHG and Cu(HG) ₂ " in Table 6.4	

Two types of titrations were performed. In the metal titrations, a mixture of the ligands (bathocuproine and glutathione) was titrated with Cu(I) varied from ca. 0.1 to 2.5×10^{-4} M for $[\text{G}]_{\text{T}} = 4.0 \times 10^{-4}$ M and $[\text{B}]_{\text{T}}$ of either 1.0 or 2.0×10^{-4} M. In the ligand titrations, a mixture of glutathione and Cu(I) was titrated with bathocuproine with $[\text{B}]_{\text{T}}$ varied from 0.1 to 1.6×10^{-4} M for $[\text{G}]_{\text{T}} = 4.0 \times 10^{-4}$ M and $[\text{Cu}]_{\text{T}} = 0.84 \times 10^{-4}$ M.

The results of the experiments are shown in Fig. 6.6; absorbance measurements made on triplicate samples agreed within $\pm 6\%$ for Cu(I) titrations and $\pm 5\%$ for bathocuproine titrations. In the earlier part of Cu(I) titrations, the presence of glutathione had no effect on formation of the CuB₂ complex; the increase in measured absorbance paralleled that observed in the absence of any competing ligand until ca. 62% of the maximum possible absorbance was achieved (for given $[\text{B}]_{\text{T}}$). However, further additions of Cu(I) did not increase the measured absorbance to its maximum possible value but instead decreased the measured absorbance substantially. Thus, at higher $[\text{Cu(I)}]_{\text{T}}$, the complex CuB₂ must lose Cu(I) to another complex that has negligible absorbance at 480 nm.

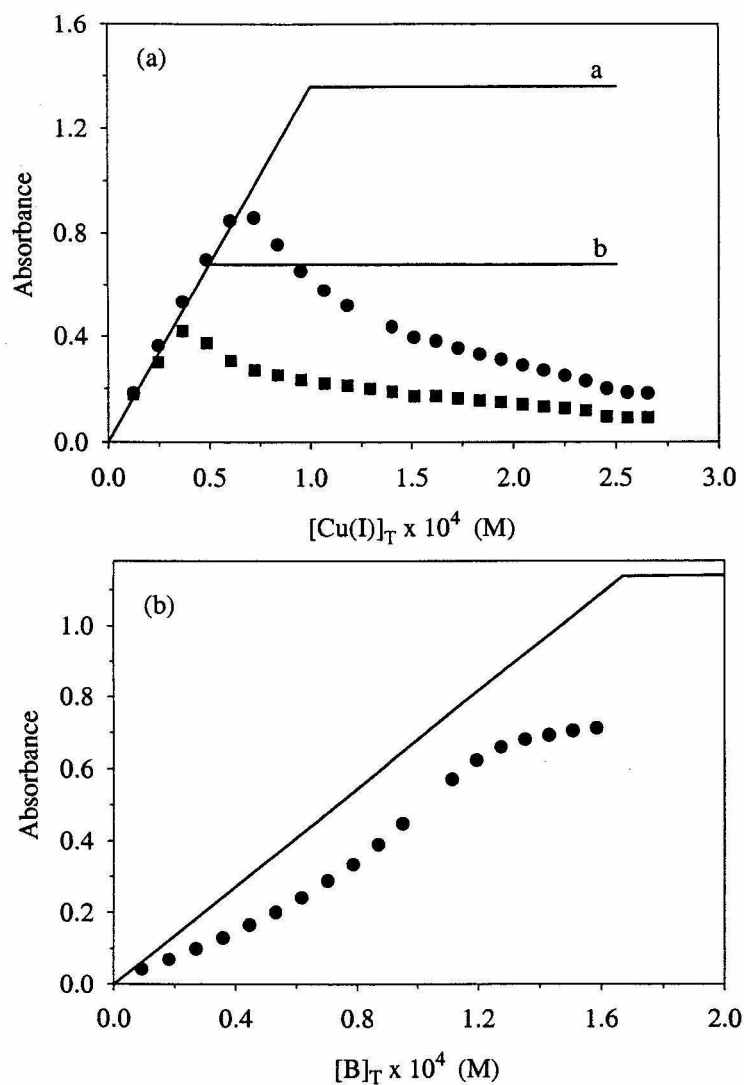


Figure 6.6. Cu(I) and bathocuproine (B) titration curves of glutathione (G).
 (a) Cu(I) titrations of $[\text{G}]_{\text{T}} = 4.0 \times 10^{-4} \text{ M}$, $[\text{B}]_{\text{T}} = 1.0 \times 10^{-4} \text{ M}$ (■) and $2.0 \times 10^{-4} \text{ M}$ (●);
 (b) Bathocuproine titrations of $[\text{G}]_{\text{T}} = 4.0 \times 10^{-4} \text{ M}$, $[\text{Cu}]_{\text{T}} = 0.84 \times 10^{-4} \text{ M}$ (●).
 Solid lines represent absorbance of CuB_2 in the absence of any competing ligand.
 Line (a): expected maximum absorbance for $[\text{B}]_{\text{T}} = 2.0 \times 10^{-4} \text{ M}$;
 line (b): expected maximum absorbance for $[\text{B}]_{\text{T}} = 1.0 \times 10^{-4} \text{ M}$.
 $\text{pH} = 6.5$, $I = 0.05 \text{ M}$.

In contrast, the observed absorbance in bathocuproine titrations was lower than that expected in the absence of glutathione over the entire range of $[B]_T$. As also observed in the Cu(I) titrations, the measured absorbance reached only ca. 62% of its maximum possible value (for given $[Cu(I)]_T$). These observations indicate that the ligands glutathione and bathocuproine compete for Cu(I) throughout the bathocuproine titration experiments.

6.5.2.1 Treatment of the titration data

Several assumptions were made throughout. Equilibrium was assumed for each titration point (see section 6.4.3). The measured absorbance at 480 nm was attributed solely to formation of the complex CuB_2 (see also section 6.5.2.6 below). Formation of 1:1 CuB complex was not considered.

For an initial evaluation of the consistency of the data sets and to determine the types of complexes needed to fit the observations, modeling of the experimental data was performed using MINEQL⁺ including only the 1:1 and 1:2 complexes of Cu(I) with glutathione (CuG and CuG_2) and the 1:2 complex with bathocuproine (CuB_2). Later other species were introduced as needed to improve the agreement of the modeling results with the data, and constants were optimized using FITEQL 4.0.

6.5.2.2 *Comparison of predicted and experimental Cu(I) speciation*

Both bathocuproine and Cu(I) titration experiments show significant disagreement with the speciation calculated using MINEQL⁺ with reported stability constants for CuG and CuG₂ complexes and the estimated stability constant for the CuB₂ complex (Fig. 6.7). Even the overall shape of the Cu(I) titration data is poorly predicted (Fig. 6.7a). The sharp decrease in [CuB₂] at higher [Cu(I)]_T, corresponding to the observed decrease in absorbance, can not be accounted for by changing glutathione competition strength. The extent of glutathione competition for Cu(I) determines the position of CuB₂ curve; for higher competition strength of glutathione, the maximum CuB₂ concentration is smaller and it is reached at lower [Cu(I)]_T. The sharp decrease in [CuB₂] can be explained only if an additional complex competing for both Cu(I) and bathocuproine is formed in the solution. To account for the experimental observations a ternary bathocuproine-Cu(I)-glutathione (BCuG) complex must be introduced.

6.5.2.3 *Plausibility of a mixed complex*

Formation of a ternary 1:1:1 complex in which Cu(I) is bound to both glutathione and bathocuproine may be expected since both bathocuproine and glutathione form 1:2 complexes of similar stability. In other studies of ternary systems with Cu(I) (specifically, Cu(I)-cysteine-penicillamine and Cu(I)-cysteine-glutathione), mixed complexes (including polynuclear complexes with two Cu(I) ions in the complex) were reported to be formed over a wide pH range (Tran-Ho et al., 1997).

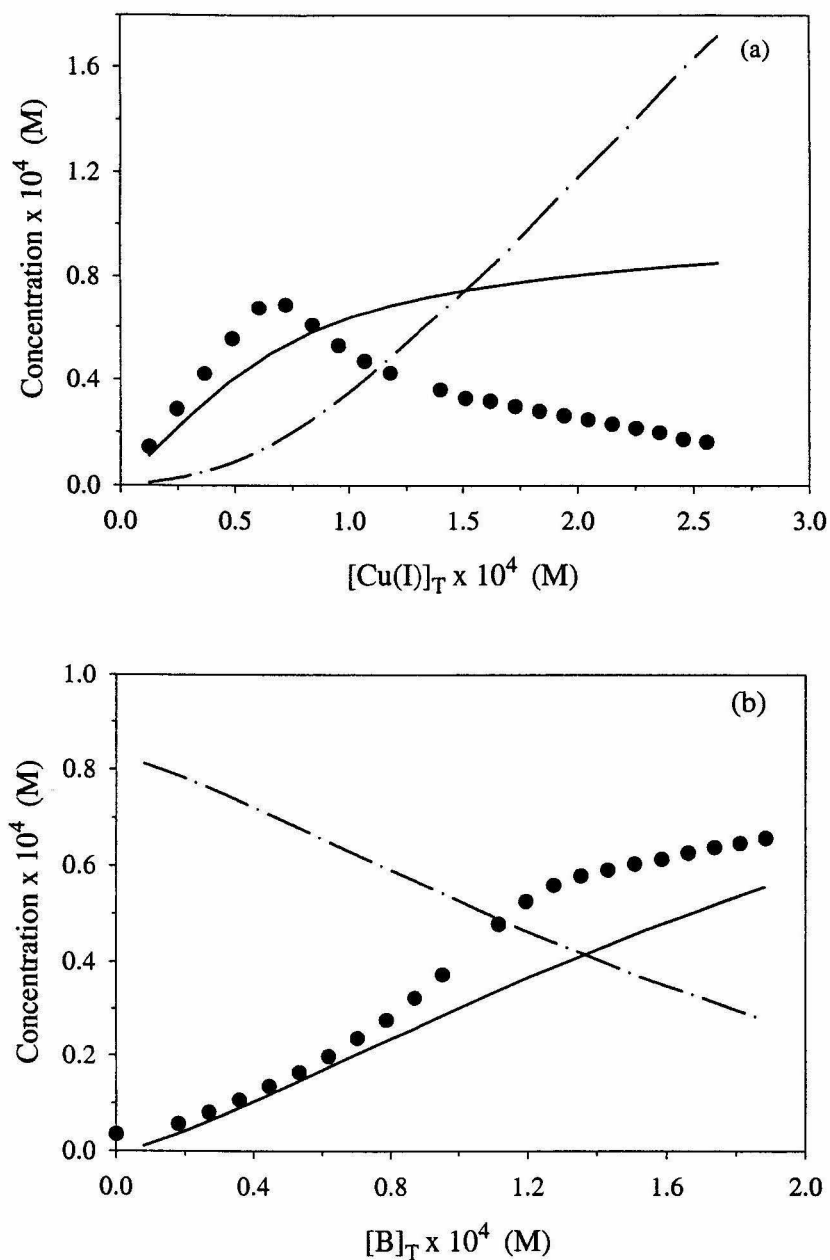


Figure 6.7. Cu(I) speciation in the presence of glutathione (G) and bathocuproine (B).
 (a) $[\text{G}]_{\text{T}} = 4.0 \times 10^{-4} \text{ M}$, $[\text{B}]_{\text{T}} = 2.0 \times 10^{-4} \text{ M}$.
 (b) $[\text{G}]_{\text{T}} = 4.0 \times 10^{-4} \text{ M}$, $[\text{Cu(I)}]_{\text{T}} = 0.84 \times 10^{-4} \text{ M}$; pH = 6.5, $I = 0.05 \text{ M}$.
 Experimental data: (●) $[\text{CuB}_2]$ calculated from measured absorbance at 480 nm.
 Model predictions (MINEQL⁺):
 (—) $[\text{CuB}_2]$, $\log \beta_{\text{CuB}_2}^{\text{cond}} = 18.5$; (- · -) $[\text{CuG}]$, $\log K_{\text{CuG}}^{\text{cond}} = 13.4$.
 Note that 1:2 CuG_2 complex is predicted to be $\leq 0.02 \times 10^{-4} \text{ M}$ under all experimental conditions.

Stabilization effects in mixed ligand complexes with 3d metals have been demonstrated for many biologically important complexes (including Cu(I)/Cu(II) complexes with N of imidazole moieties and S of sulfhydryl groups) (Sigel et al., 1977). Ternary copper(II)-phenanthroline-amino acids complexes are also common (Antolini et al., 1985, Fischer and Sigel, 1980, and Gergely et al. 1972). In the case of Cu(II) complexes with oxidized glutathione and five differently substituted 1,10-phenanthrolines, it has been suggested that the ternary 1:1:1 phenanthroline-Cu(II)-oxidized glutathione complex is the most stable species in the pH range from 4 to 8 (Piu et al., 1997). This should not, however, be taken to imply the formation of a similar ternary bathocuproine-Cu(II)-oxidized glutathione complex in the present study which was performed under deoxygenated conditions with addition of Cu(I). No signal of the oxidized complex (which would be observed at ca. 600 nm) was observed when absorbance spectra were acquired from 200 - 800 nm.

6.5.2.4 *Inclusion of a ternary (BCuG) complex in modeling experimental data*

Introduction of a ternary BCuG complex can account for the decrease in the concentration of CuB₂ at higher Cu(I) concentrations (Fig. 6.8 a, c). However, the obtained fit is poor in the earlier part of the titration curve (at lower [Cu(I)]_T). Optimization of the ternary complex stability constant for Cu(I) titration data using the reported/estimated constants for CuG, CuG₂ and CuB₂ gives $\log \beta_{BCuG}^{cond} = 18.0$ for [B]_T = 2.0 and $\log \beta_{BCuG}^{cond} = 17.8$ for [B]_T = 1.0 x 10⁻⁴ M (Table 6.6). The fit may be improved if conditional stability constants for both CuG and BCuG are optimized (Fig 6.8 b, d).

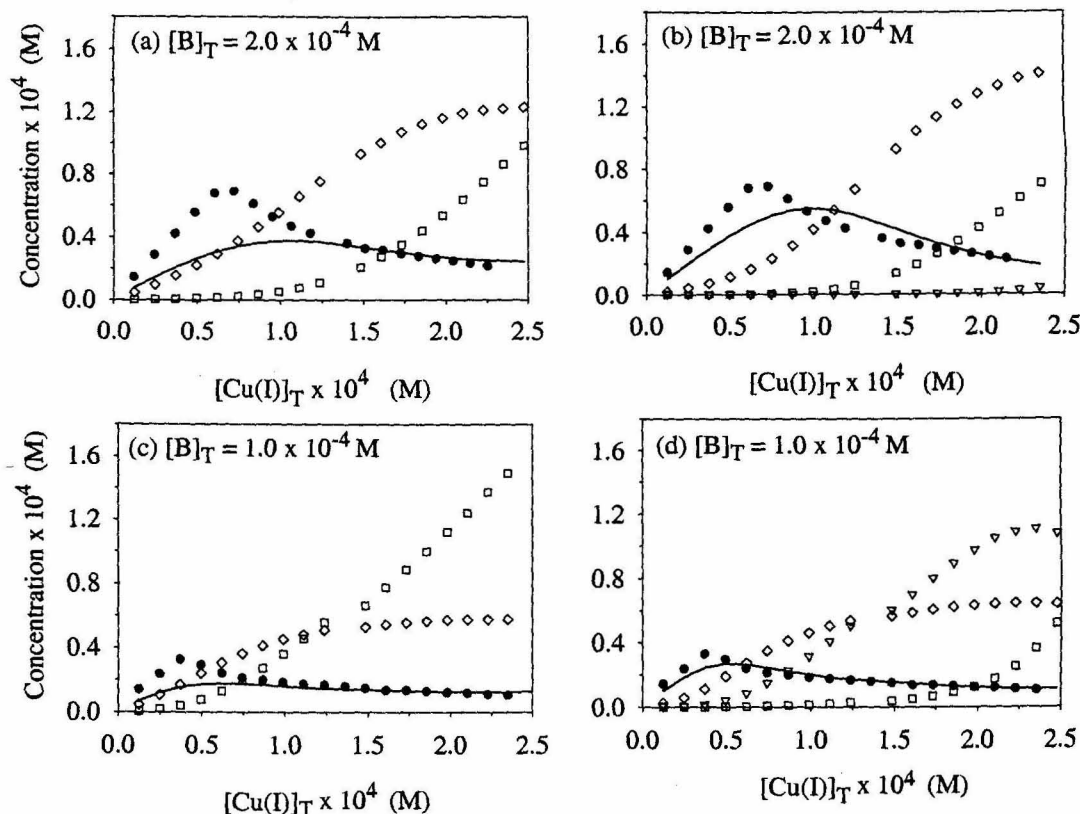


Figure 6.8. Cu(I) speciation in Cu(I) titrations of glutathione (G) and bathocuproine (B) obtained using FITEQL 4.0.
 (a, c) Fits obtained by optimizing conditional stability constant of BCuG.
 (b, d) Fits obtained by optimizing conditional stability constant of BCuG and CuG.
 For the values of the constants see Table 6.6.
 Conditions: [G]_T = 4.0 x 10⁻⁴ M,
 (a, b) [B]_T = 2.0 x 10⁻⁴ M, (c, d) [B]_T = 1.0 x 10⁻⁴ M; pH = 6.5, *I* = 0.05 M.
 Symbols: (●) [CuB₂] calculated from measured absorbance at 480 nm.
 Model predictions: (—) [CuB₂], (□) [CuG], (◇) [BCuG], (▽) [CuG₂].
 Note that in (a, c) CuG₂ is predicted to be < 0.01 x 10⁻⁴ M.

The optimized value of the constant for CuG is smaller than the literature value (10^{9.9} and 10^{10.3} vs. 10^{13.4}), which leads to less competition from that complex in the later part of the titration curve. Note that the concentration of CuG in the beginning of the titration is negligible. With a weaker CuG complex, a lower stability can be assigned to

the ternary complex and still maintain competition with the CuG complex at higher $[\text{Cu(I)}]_{\text{T}}$. A weaker BCuG complex competes less with CuB_2 at lower $[\text{Cu(I)}]_{\text{T}}$, which improves the fit in the beginning of the titration curves.

The optimized values of the conditional stability constants for the CuG and BCuG complexes obtained in two Cu(I) titration experiments are consistent. However, the constant obtained for the CuG complex is three orders of magnitude smaller than its reported value and two orders of magnitude smaller than the value obtained from the bathocuproine titrations. It can be seen in Fig. 9c that the $[\text{CuB}_2]$ predicted for bathocuproine titrations with MINEQL⁺ using $\log K_{\text{CuG}}^{\text{cond}} = 10.1$ and $\log \beta_{\text{BCuG}}^{\text{cond}} = 17.3$ obtained in Cu(I) titrations deviates substantially from the measured values (Fig. 6.9c). However, this deviation mainly results from the difference in the strength of the mixed complex ($10^{17.3}$ vs. $10^{16.7}$). The fit shows low sensitivity to the value of the conditional stability constant for the CuG complex. Thus in the same optimized value for $\log \beta_{\text{BCuG}}^{\text{cond}} = 16.7$ is obtained using $\log K_{\text{CuG}}^{\text{cond}} = 10.1$, as obtained in Cu(I) titrations, or $\log K_{\text{CuG}}^{\text{cond}} = 12.4$, as obtained in bathocuproine titrations (Table 6.6). It can be seen in Fig. 6.9b that the fit is minimally affected by the difference in the value of $\log K_{\text{CuG}}^{\text{cond}}$.

Table 6.6. Optimization of conditional stability constants (pH = 6.5, $I = 0.05$) for Cu(I) and bathocuproine (B) titrations of glutathione					
titrant	experimental conditions	species with fixed constants		species with optimized constants	
		species	$\log \beta_{CuL_x}^{cond}$	species	$\log \beta_{CuL_x}^{cond}$
Cu(I)	[G] _T = 4.0×10^{-4} M [B] _T = 2.0×10^{-4} M	CuG	13.4	BCuG	18.0
		CuG ₂	15.3		
		CuB ₂	18.5		
Cu(I)	[G] _T = 4.0×10^{-4} M [B] _T = 2.0×10^{-4} M	CuG ₂	15.3	BCuG	17.4
		CuB ₂	18.5	CuG	9.9
Cu(I)	[G] _T = 4.0×10^{-4} M [B] _T = 1.0×10^{-4} M	CuG	13.4	BCuG	17.8
		CuG ₂	15.3		
		CuB ₂	18.5		
Cu(I)	[G] _T = 4.0×10^{-4} M [B] _T = 1.0×10^{-4} M	CuG ₂	15.3	BCuG	17.1
		CuB ₂	18.5	CuG	10.3
B	[G] _T = 4.0×10^{-4} M [Cu] _T = 0.84×10^{-4} M	CuG	13.4	BCuG	no convergence
		CuG ₂	15.3		
		CuB ₂	18.5		
B	[G] _T = 4.0×10^{-4} M [Cu] _T = 0.84×10^{-4} M	CuG ₂	15.3	BCuG	16.7
		CuB ₂	18.5	CuG	12.4
B	[G] _T = 4.0×10^{-4} M [Cu] _T = 0.84×10^{-4} M	CuG	10.1	BCuG	16.7
		CuG ₂	15.3		
		CuB ₂	18.5		

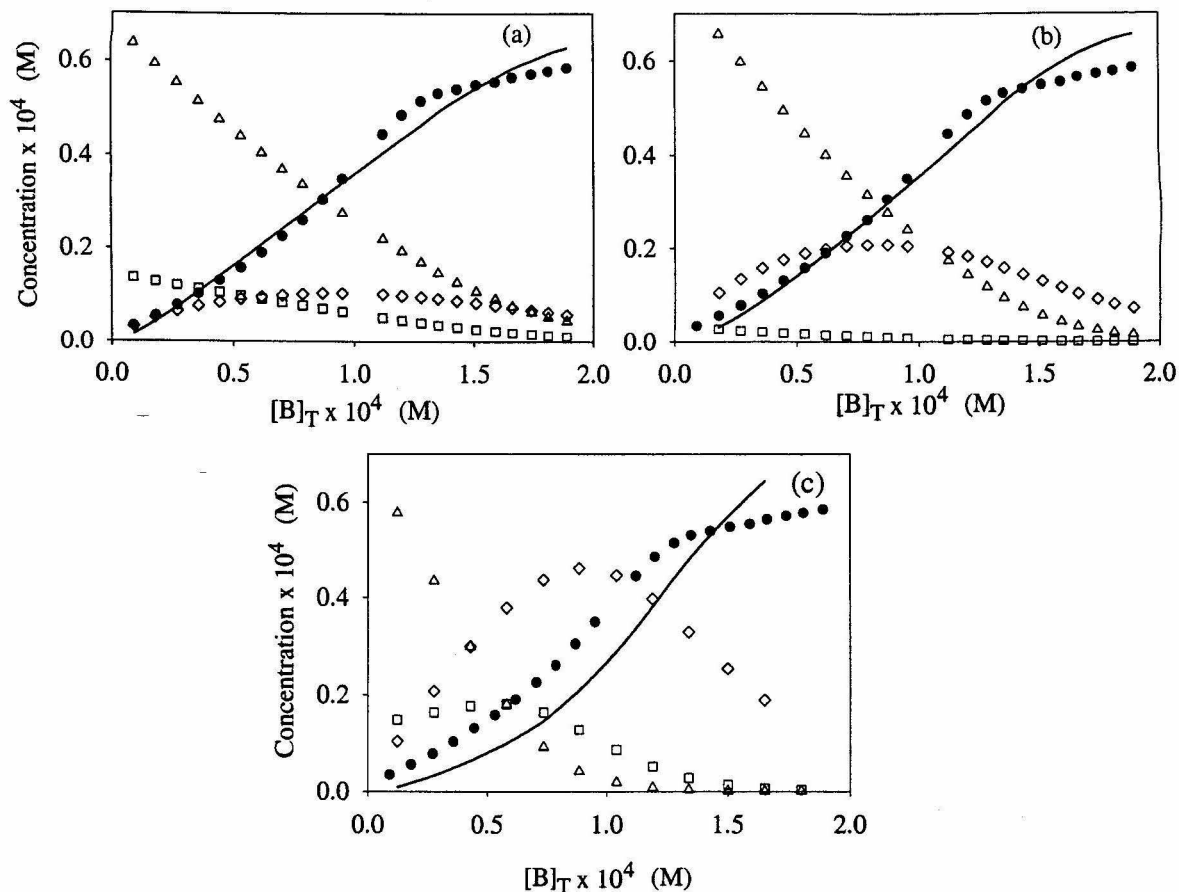


Figure 6.9. Cu(I) speciation in bathocuproine (B) titrations of glutathione (G) obtained using FITEQL 4.0.
 (a) Fit obtained by optimizing conditional stability constants of BCuG and CuG.
 (b) Fit obtained by optimizing conditional stability constant of BCuG and fixed CuG optimized in Cu(I) titration experiments.
 (c) Model predictions (MINEQL⁺) based on BCuG and CuG conditional stability constants obtained in Cu(I) titration experiments.
 For the values of the constants see Table 6.6.
 Conditions: $[G]_T = 4.0 \times 10^{-4} \text{ M}$, $[Cu]_T = 0.84 \times 10^{-4} \text{ M}$, $\text{pH} = 6.5$, $I = 0.05 \text{ M}$
 Symbols: (●) $[CuB_2]$ calculated from the measured absorbance at 480 nm.
 Model predictions: (—) $[CuB_2]$, (Δ) $[CuG]$, (\diamond) $[BCuG]$, (\square) $[CuG_2]$.

It is possible that discrepancies between conditional stability constants obtained in Cu(I) titration experiments and those reported in the literature and obtained in bathocuproine titration experiments may reflect kinetic artifacts related to conditions allowing a

local excess of Cu(I) and glutathione. In bathocuproine titrations, Cu(I) and glutathione were pre-mixed by one time addition of the reagents from stock solutions. In the prior determination of the stability constants for Cu(I)-glutathione, Cu(I) ions were generated into the ligand solution by constant-current electrolysis of a two-phase amalgam. Under these conditions, Cu(I) ions are likely to be in excess in the vicinity of the amalgam. There is also likely to be a local excess of glutathione due to its adsorption on the electrode (Le Gall and van den Berg, 1998).

6.5.2.5 *Evaluation of obtained results*

The value obtained for the stability constant of the mixed complex may be evaluated based on the donor group additivity approach (Hancock and Martell, 1989). This approach uses a "rule of average environment" in which the formation constant of a ligand containing two different types of donor groups is taken to be the average of the formation constants of a pair of similar ligands each containing only one of the two donor types. For example, accurate stability constant predictions were made for Ca^{2+} , In^{3+} and Pb^{2+} complexes of 8-hydroxyquinoline based on the average of their stability constants with 1,10-phenanthroline and catechol. In case of the mixed BCuG complex, the average of the stability constants for the CuB_2 and CuG_2 gives $\log \beta_{\text{BCuG}}^{\text{cond}} = 16.8$. This value is somewhat smaller than that obtained by fitting Cu(I) titration experiments ($\log \beta_{\text{BCuG}}^{\text{cond}} \approx 17.3$).

6.5.2.6 Possible interference of the mixed complex

The preceding optimization of stability constants was based on the assumption that absorbance of the ternary complex at 480 nm is negligible. This assumption can be shown to be reasonable by examining the Cu(I) titration data. The bathocuproine titration data are not considered since the ternary complex formation is predicted to be less significant in these experiments.

Since the BCuG concentration is predicted to be at its maximum at the end of Cu(I) titration curve (Fig. 6.8), the contribution of BCuG to the absorbance measured throughout the titration can not be greater than the absorbance observed for the last few titration points. In order to account for the decreasing absorbance at higher $[\text{Cu(I)}]_{\text{T}}$ by formation of BCuG at the expense of CuB_2 , ϵ_{BCuG} must be less than ϵ_{CuB_2} . If some of the observed absorbance is due to the mixed complex, then the actual $[\text{CuB}_2]$ would be smaller than that included in the modeling and, as a result, competition from CuB_2 would also be smaller. If true, this must apply at the beginning as well as the end of the titration curve. Less competition from CuB_2 would lead to higher concentrations of BCuG and, since ϵ_{BCuG} is less than ϵ_{CuB_2} , would decrease the observed absorbance. Comparison of the predicted and observed absorbance (Fig. 6.8) indicates that the model already underpredicts the absorbance in the earlier part of the titration curve, so including a finite ϵ_{BCuG} would only make the fit worse.

This qualitative argument may be confirmed by modeling the experimental data with a non-zero value of the molar extinction coefficient. For a non-zero ϵ_{BCuG} , the observed absorbance of the CuB_2 is given by

$$\text{Abs}_{\text{exp}} = \epsilon_{\text{CuB}_2} [\text{CuB}_2] + \epsilon_{\text{BCuG}} [\text{BCuG}]$$

and

$$\frac{\text{Abs}_{\text{exp}}}{\epsilon_{\text{CuB}_2}} = [\text{CuB}_2] + \frac{\epsilon_{\text{BCuG}}}{\epsilon_{\text{CuB}_2}} [\text{BCuG}].$$

Then for FITEQL modeling a dummy variable d is introduced, where

$$d = \left(\frac{\text{Abs}_{\text{exp}}}{\epsilon_{\text{CuB}_2}} \right)$$

and the experimental data are input as serial data for the component d for each $[\text{Cu(I)}]_{\text{T}}$.

In addition the coefficient $\frac{\epsilon_{\text{BCuG}}}{\epsilon_{\text{CuB}_2}}$ is included for the species BCuG in the mass balance matrix.

The extinction coefficient for the mixed complex can not be more than 10% of ϵ_{CuB_2} . Even if all the absorbance observed at the last few titration points is assumed to be due to the BCuG complex ($[\text{BCuG}]_{\text{max}} = 1.4 \times 10^{-4} \text{ M}$), ϵ_{BCuG} is only 10% of ϵ_{CuB_2} .

Optimization of $\log \beta_{\text{CuL}_x}^{\text{cond}}$ of the CuG and BCuG complexes with a non-zero extinction coefficient of the mixed complex was performed for $[\text{G}]_{\text{T}} = 4.0 \times 10^{-4} \text{ M}$ and $[\text{B}]_{\text{T}} = 1.0 \times 10^{-4} \text{ M}$ (Table 6.7). The optimization procedure converged with up to the 5% of the total absorbance been ascribed to the mixed complex. As the contribution of the mixed complex to the measured absorbance increased, the optimized value of the condi-

tional stability constant of the CuG complex decreased. Inclusion of a finite extinction coefficient for BCuG thus yields a stability constant for the complex CuG which is even further from that found in the literature than the constant obtained neglecting any absorbance due to the ternary complex. These arguments suggest that interference of the ternary complex with the absorbance measurements at 480 nm can be neglected.

Table 6.7. Optimization of conditional stability constants (pH = 6.5, $I = 0.05$) of the CuG and BCuG complexes using a non-zero mixed complex extinction coefficient		
$\left(\frac{\epsilon_{\text{BCuG}}}{\epsilon_{\text{CuB}_2}} \right) * 100 \%$	Optimized constants, $\log \beta_{\text{CuL}_x}^{\text{cond}}$	
	CuG	BCuG
0	10.30	17.10
1	10.16	17.14
3	9.74	17.17
5	8.70	17.21

6.5.2.7 Concluding remarks

The experiments with glutathione illustrate the applicability of the spectroscopic technique with bathocuproine for determination of conditional stability constants of Cu(I) complexes with thiol-containing ligands. The results of the Cu(I) titration experiments for the two different concentrations of bathocuproine agree well. The conditional stability constants obtained for the CuG complex in these experiments is weaker than that obtained in the bathocuproine titrations (which is closer to the previously reported con-

stant). However the conditions of these latter experiments may allow for a local excess of Cu(I) and glutathione which would not be representative of the natural conditions under which copper is taken up by a living cell.

6.5.3 Investigation of Cu(I)-phytochelatin complexation using a spectroscopic technique with bathocuproine -

Three series of titrations were performed to obtain conditional stability constants of Cu(I) complexes with phytochelatin: titrations of phytochelatin/bathocuproine with Cu(I), titrations of Cu(I)/bathocuproine with phytochelatin and titrations of phytochelatin/Cu(I) with bathocuproine. The conditions of these titration experiments are summarized in Table 6.8. Note that, in these experiments, the phytochelatin concentration was a factor of four lower than the concentration of glutathione used in the experiments described in section 6.5.2. The results of the experiments are shown in Fig. 6.10; the absorbance measurements made on triplicate samples agreed within $\pm 6\%$ for phytochelatin and bathocuproine experiments and $\pm 5\%$ for Cu(I) titrations.

Table 6.8. Summary of experimental conditions for Cu(I) bathocuproine (B) and phyto-chelatin (P) titrations (pH = 6.5, $I = 0.05$)			
titrant	[Cu] x 10⁴ M	[P] x 10⁴ M	[B] x 10⁴ M
Cu(I)	0.12 - 3.2	1.0	1.0 2.0
P	1.0	0.14 - 1.4	1.0
B	1.0 1.4	1.0	0.5 - 2.0

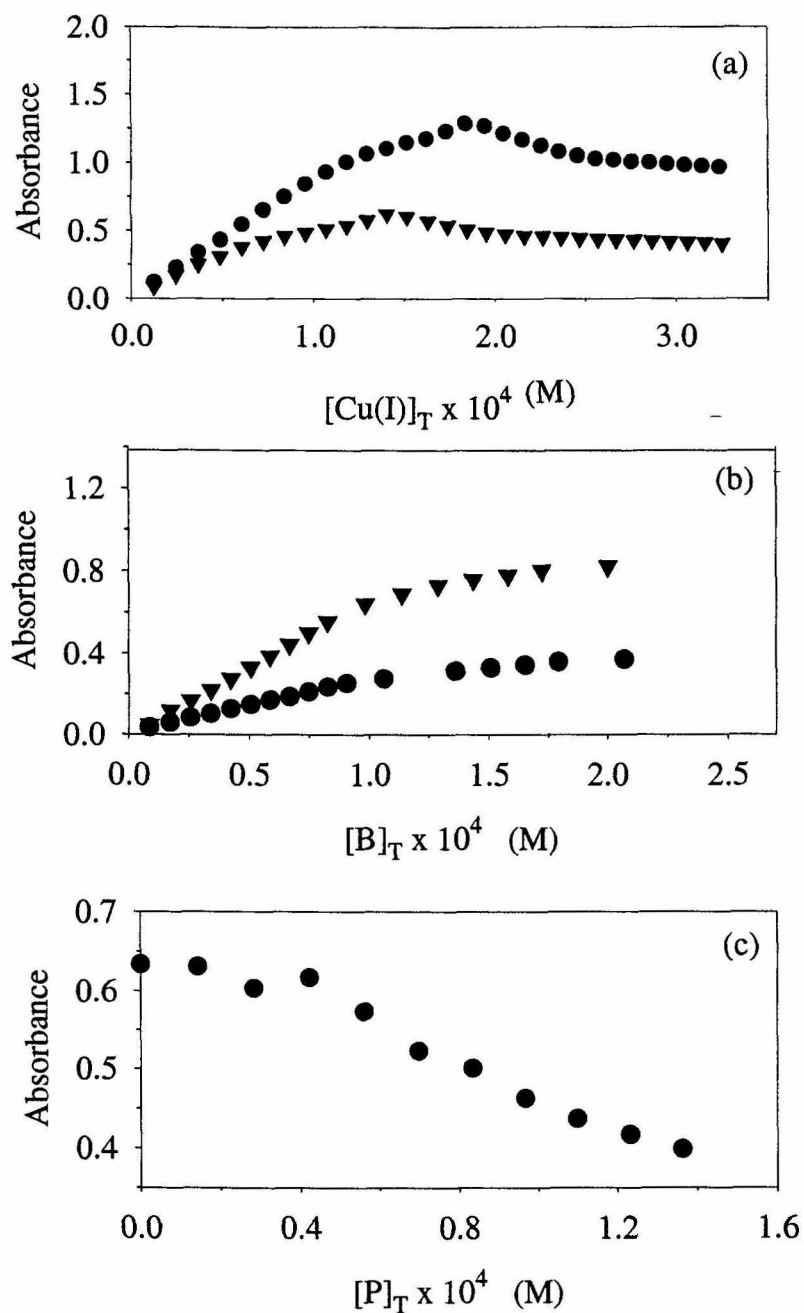


Figure 6.10. Titration curves for phytochelatin (P).
 (a) Copper titrations of $[P]_T = 1.0 \times 10^{-4} \text{ M}$, $[B]_T = 1.0$ (▼) and 2.0 (●) $\times 10^{-4} \text{ M}$.
 (b) Bathocuproine titrations of $[P]_T = 1.0 \times 10^{-4} \text{ M}$, $[Cu]_T = 1.4$ (▼) and 1.0 (●) $\times 10^{-4} \text{ M}$.
 (c) Phytochelatin titrations of $[B]_T = 1.0 \times 10^{-4} \text{ M}$, $[Cu]_T = 1.0 \times 10^{-4} \text{ M}$ (●).
 $\text{pH} = 6.5$, $I = 0.05 \text{ M}$.

6.5.3.1 *Cu titration experiments*

Cu(I) titration data of phytochelatin and $[B]_T$ at either 1.0 or 2.0×10^{-4} M are shown in Fig. 6.11. The solid lines in the figure show the expected absorbance of CuB_2 in the absence of any competing ligand. These absorbance curves are initially identical for the two $[B]_T$ values, but the maximum absorbance is smaller for the lower $[B]_T$ (i.e., $Abs_{max}^1 < Abs_{max}^2$). In the beginning of the titrations, the experimental data lie below the solid lines. The maximum possible absorbance of CuB_2 (i.e., corresponding to that in the absence of any competing ligand) is, however, reached for both $[B]_T$. At higher $[Cu]_T$, the absorbance decreases from the maximum values.

These various features of the titration curves are indicative of the types of complexes formed during the titration. Specifically, the values of $[Cu]_T$ at which the maximum absorbances are observed ($[Cu]_{A(1)max}$ and $[Cu]_{A(2)max}$) suggest the presence of both 1:1 and 1:2 copper-phytochelatin complexes. For example, for $[B]_T = 2.0 \times 10^{-4}$ M, the maximum possible concentration of the CuB_2 complex is 1.0×10^{-4} M (or $1/2 [B]_T$). The maximum absorbance corresponding to this concentration is observed at $[Cu]_T = 1.8 \times 10^{-4}$ M. If all copper in excess of that bound in CuB_2 is complexed by phytochelatin either as a 1:1 or 1:2 complex, then

$$[CuP] + [CuP_2] = [Cu]_{A(2)max} - 1/2 [B]_T = 0.8 \times 10^{-4} \text{ M.}$$

Since $[P]_T = 1.0 \times 10^{-4}$ M, not all of the Cu(I) bound by phytochelatin can be present as CuP_2 . Thus, both CuP and CuP_2 are present in the solution.

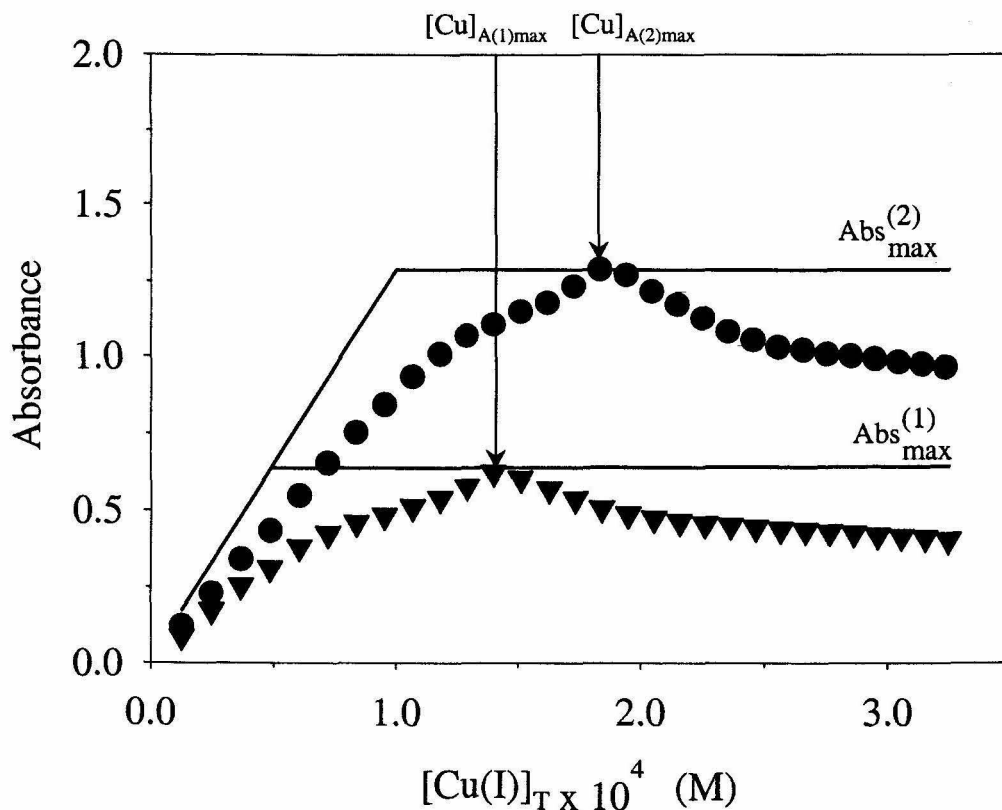


Figure 6.11. Cu(I) titration curves of phytochelatin (P) and bathocuproine (B).
 Conditions:
 $[P]_T = 1.0 \times 10^{-4}$ M, $[B]_T = 1.0 \times 10^{-4}$ (▼) and 2.0×10^{-4} (●) M.
 See the text for the explanations of $Abs_{max}^{(1)}$, $Abs_{max}^{(2)}$ lines and
 $[Cu]_{A(1)max}$, $[Cu]_{A(2)max}$.
 $pH = 6.5$, $I = 0.05$ M.

The decrease in absorbance from its maximum value toward the end of the titrations is indicative of a mixed complex formation. A similar decrease in the absorbance observed in Cu(I) titration experiments of glutathione was attributed to the formation of a

ternary BCuG complex (see section 6.5.2.1). Since the maximum possible absorbance is reached during the titrations (line $\text{Abs}_{\text{max}}^{(1)}$ for $[\text{B}]_{\text{T}} = 1.0 \times 10^{-4} \text{ M}$ or line $\text{Abs}_{\text{max}}^{(2)}$ for $[\text{B}]_{\text{T}} = 2.0 \times 10^{-4} \text{ M}$), the mixed complex can not be present in the beginning of the curve (i.e., $[\text{Cu}]_{\text{T}} < [\text{Cu}]_{\text{A}(1)\text{max}}$ for $[\text{B}]_{\text{T}} = 1.0 \times 10^{-4} \text{ M}$ or $[\text{Cu}]_{\text{T}} < [\text{Cu}]_{\text{A}(2)\text{max}}$ for $[\text{B}]_{\text{T}} = 2.0 \times 10^{-4} \text{ M}$). Therefore, the decreased absorbance in the beginning of the titration curve must be due to the competition between CuB_2 and the complexes of Cu(I) with phytochelatin.

Based on this reasoning, optimization of conditional stability constants with FITEQL 4.0 was performed separately for the ascending and descending parts of the titration curves. Since the mixed complex is not present in the earlier part of the titration (e.g., $[\text{Cu}]_{\text{T}} < [\text{Cu}]_{\text{A}(1)\text{max}}$ for $[\text{B}]_{\text{T}} = 1.0 \times 10^{-4} \text{ M}$), that portion of the titration curve was used to optimize the constants for CuP and CuP_2 . These constants were then used (as fixed values) to obtain the constants for the mixed complex by fitting the later portion of the titration curves (e.g., $[\text{Cu}]_{\text{T}} > [\text{Cu}]_{\text{A}(1)\text{max}}$ for $[\text{B}]_{\text{T}} = 1.0 \times 10^{-4} \text{ M}$). Using these fixed values of the conditional stability constants for CuP and CuP_2 , the later part of the curve could be reproduced only with a polynuclear complex BCu_2P . This is consistent with the observation that the decrease in the absorbance from its maximum value was observed at higher $[\text{Cu}]_{\text{T}}$ than in glutathione titrations. Optimized constants are listed in Table 6.9.

The data of the two titrations with different bathocuproine concentrations agree reasonably well. The geometric means of the conditional stability constants obtained for the two bathocuproine concentrations are:

$$\log K_{\text{CuP}}^{\text{cond}} = 13.8 \pm 0.2, \log \beta_{\text{CuP}_2}^{\text{cond}} = 18.5 \pm 0.3, \log \beta_{\text{BCu}_2\text{P}}^{\text{cond}} = 27.0 \pm 0.3.$$

The obtained fit of the experimental data is shown in Fig. 6.12.

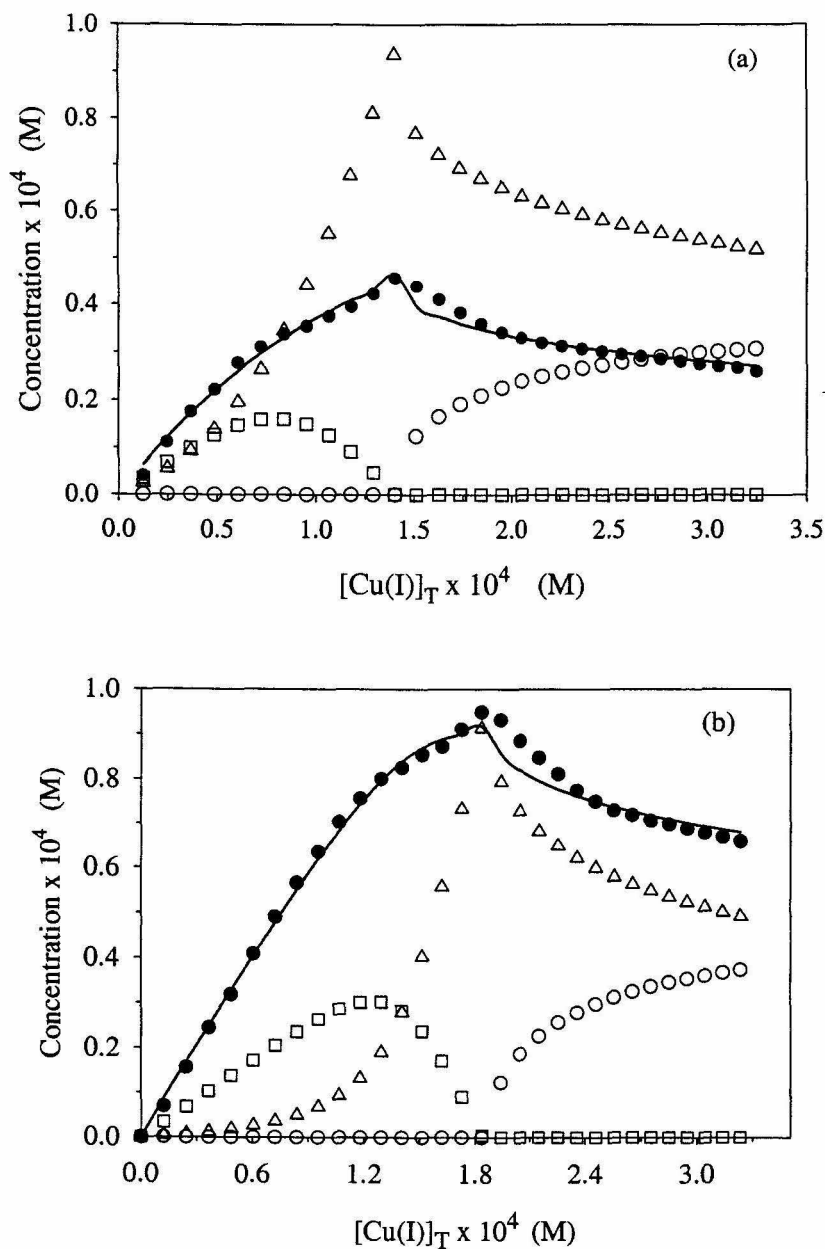


Figure 6.12. Cu(I) speciation in Cu(I) titrations of phytochelatin (P) and bathocuproine (B) obtained using FITEQL 4.0.
 (a) [P]_T = 1.0 x 10⁻⁴ M; [B]_T = 1.0 x 10⁻⁴ M.
 (b) [P]_T = 1.0 x 10⁻⁴ M; [B]_T = 2.0 x 10⁻⁴ M.
 Symbols: (●) [CuB₂] calculated from measured absorbance at 480 nm.
 For the values of the constants see Table 6.9.
 Model predictions:
 (—) [CuB₂], (△) [CuP], (○) [BCu₂P], (□) [CuP₂].
 pH = 6.5, I = 0.05 M.

6.5.3.2 *Phytochelatin titration experiments*

For the initial conditions of the phytochelatin titration experiments ($[\text{Cu}]_{\text{T}} = [\text{B}]_{\text{T}} = 1.0 \times 10^{-4} \text{ M}$), $[\text{Cu}]_{\text{T}}$ is in excess (by $0.5 \times 10^{-4} \text{ M}$) of that needed to form the CuB_2 complex. Thus additions of phytochelatin allow CuP to form without competing with CuB_2 . After the excess of copper is titrated (at $[\text{P}]_{\text{T}} = 0.5 \times 10^{-4} \text{ M}$), a decrease in $[\text{CuB}_2]$ is observed (Fig. 6.13) indicating competition for Cu(I) between the added phytochelatin and bathocuproine. As a result of this competition, formation of the CuP complex followed by the formation of CuP_2 may be expected. These qualitative expectations are confirmed numerically by using MINEQL^+ with the stability constants obtained from Cu(I) titration experiments. As a result of phytochelatin competition with CuB_2 , initial formation of the CuP complex is predicted by the model; this is followed by the decrease in $[\text{CuP}]$ as the CuP_2 complex is formed by binding added P with the CuP at higher $[\text{P}]_{\text{T}}$.

The experimental curve is slightly lower than the predicted one. This difference corresponds to the discrepancy between the conditional stability constant for the CuP_2 complex obtained with FITEQL 4.0 by fitting the phytochelatin and Cu(I) titration data ($10^{18.5}$ vs. $10^{19.1}$ respectively). The values of conditional stability constants of the CuP_2 and BCu_2P complexes obtained in Cu(I) and phytochelatin experiments agree within the experimental error. The optimized value of the conditional stability constant of the CuP complex agrees well with that obtained in Cu(I) titrations (Table 6.9).

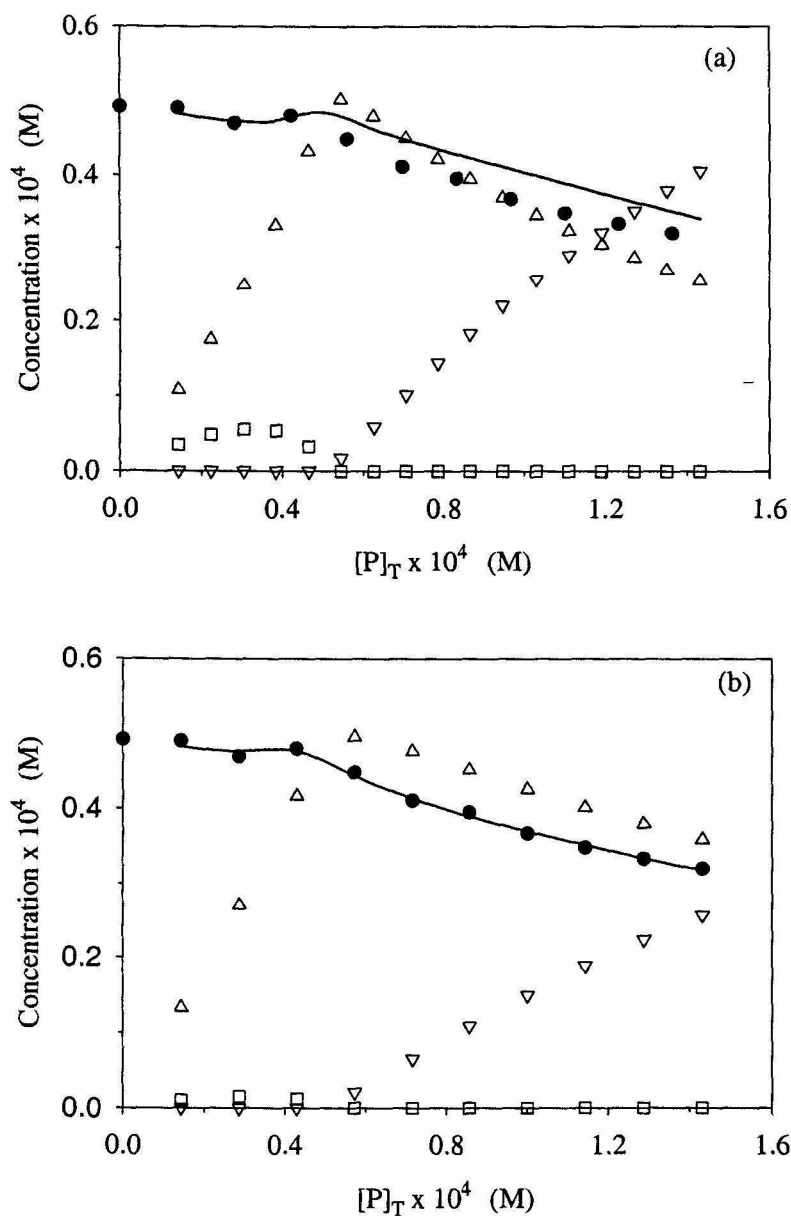


Figure 6.13. Cu(I) speciation in phytochelatin (P) titrations of Cu(I) and bathocuproine (B). $[\text{Cu(I)}]_{\text{T}} = [\text{B}]_{\text{T}} = 1.0 \times 10^{-4} \text{ M}$; $\text{pH} = 6.5$, $I = 0.05 \text{ M}$. Symbols: (●) $[\text{CuB}_2]$ calculated from measured absorbance at 480 nm, (—) $[\text{CuB}_2]$, (▽) $[\text{CuP}_2]$, (Δ) $[\text{CuP}]$, (□) $[\text{BCu}_2\text{P}]$; (a) Model predictions (MINEQL⁺) based on stability constants obtained in Cu(I) titration experiments. (b) Model predictions (FITEQL 4.0) based on optimized values of conditional stability constants. For the values of the constants see Table 6.9.

Table 6.9. Optimization of conditional stability constants (pH = 6.5, $I = 0.05$) for Cu(I) and phytochelatin (P) titrations

titrant (titration range)	experimental conditions	species with fixed constants		species with optimized constants	
		species	$\log \beta_{CuL_x}^{cond}$	species	$\log \beta_{CuL_x}^{cond}$
$[Cu(I)]_T = (0.12 - 1.8) \times 10^{-4} \text{ M}$	$[P]_T = 1.0 \times 10^{-4} \text{ M}$ $[B]_T = 2.0 \times 10^{-4} \text{ M}$	CuB_2	18.5	CuP	13.6
				CuP_2	18.7
$[Cu(I)]_T = (1.9 - 3.2) \times 10^{-4} \text{ M}$	$[P]_T = 1.0 \times 10^{-4} \text{ M}$ $[B]_T = 2.0 \times 10^{-4} \text{ M}$	CuP_2	18.69	BCu_2P	26.7
		CuB_2	18.5		
		CuP	13.61		
$[Cu(I)]_T = (0.12 - 1.4) \times 10^{-4} \text{ M}$	$[P]_T = 1.0 \times 10^{-4} \text{ M}$ $[B]_T = 1.0 \times 10^{-4} \text{ M}$	CuB_2	18.5	CuP	14.0
				CuP_2	18.2
$[Cu(I)]_T = (1.5 - 3.2) \times 10^{-4} \text{ M}$	$[P]_T = 1.0 \times 10^{-4} \text{ M}$ $[B]_T = 1.0 \times 10^{-4} \text{ M}$	CuP_2	18.23	BCu_2P	27.2
		CuB_2	18.5		
		CuP	14.02		
$[P]_T = (0.14 - 1.4) \times 10^{-4} \text{ M}$	$[Cu]_T = 1.0 \times 10^{-4} \text{ M}$ $[B]_T = 1.0 \times 10^{-4} \text{ M}$	CuB_2	18.5	CuP	13.8
				CuP_2	17.9
				BCu_2P	26.7
$[B]_T = (0.19 - 2.1) \times 10^{-4} \text{ M}$	$[Cu]_T = 1.0 \times 10^{-4} \text{ M}$ $[P]_T = 1.0 \times 10^{-4} \text{ M}$	CuB_2	18.5	CuP	16.3
				CuP_2	21.2

6.5.3.3 Bathocuproine titration experiments

For the initial conditions of the two bathocuproine titration experiments, Cu(I) and phytochelatin are present in equimolar concentrations in one case ($[Cu]_T = [P]_T = 1.0 \times 10^{-4} \text{ M}$) (Fig. 6.14b). In the other, $[Cu]_T$ is in excess (by $0.4 \times 10^{-4} \text{ M}$) of that needed to

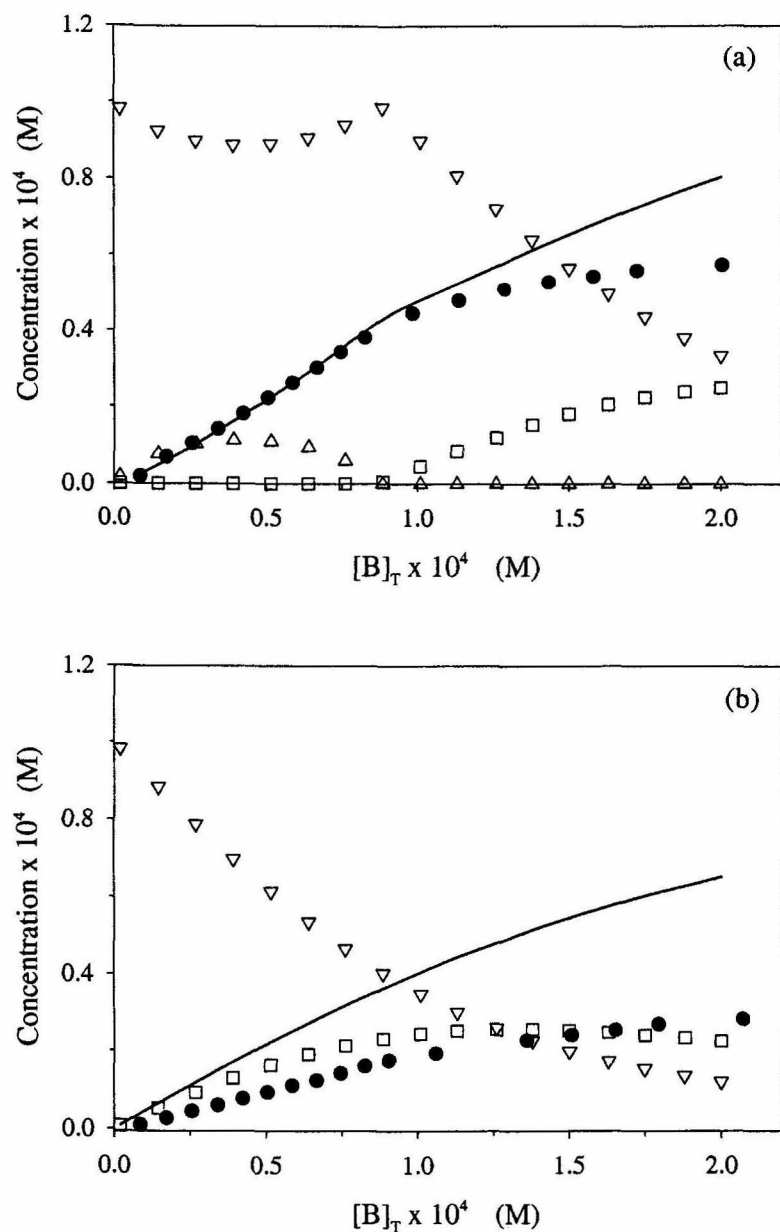


Figure 6.14. Cu(I) speciation in bathocuproine (B) titrations of Cu(I) and phytochelatin based on the conditional stability constants obtained in Cu(I) titration experiments. $[P]_T = 1.0 \times 10^{-4}$ M, $[Cu]_T = 1.4 \times 10^{-4}$ M (a), 1.0×10^{-4} M (b), pH = 6.5, $I = 0.05$ M. Experimental data: (●) $[CuB_2]$ calculated from measured absorbance at 480 nm. Model predictions (MINEQL⁺): (—) $[CuB_2]$, (Δ) $[BCu_2P]$, (∇) $[CuP]$, (\square) $[CuP_2]$. For the values of the constants see Table 6.9. Note that in (b) $[BCu_2P]$ complex is predicted to be $< 0.01 \times 10^{-4}$ M.

form CuP complex ($[Cu]_T = 1.4 \times 10^{-4} \text{ M}$ and $[P]_T = 1.0 \times 10^{-4} \text{ M}$) (Fig. 6.14a). In the latter case, the observed $[CuB_2]$ is very close to the concentration expected in the absence of competing ligands until the excess of copper is titrated (i.e., for $[B]_T < 0.8 \times 10^{-4} \text{ M}$). The slight deviation can be attributed to the formation of small amounts of the polynuclear PCu_2B complex. After the excess of copper is titrated, the increase in the measured absorbance deviates from linearity indicating competition between the added bathocuproine and the CuP complex. For $[Cu]_T = 1.0 \times 10^{-4} \text{ M}$, the measured absorbance lies lower than that expected in the absence of a competing ligand over the entire range of $[B]_T$.

The MINEQL⁺ modeling of bathocuproine titration experiments for the stability constants obtained in Cu(I) titrations (also shown in Fig. 6.14) suggests that, as bathocuproine is added, the Cu(I) is lost from CuP to CuB_2 . As a consequence, phytochelatin is released, and is available to form CuP_2 . When CuP is mostly titrated, CuB_2 starts competing with CuP_2 . Competition of the added bathocuproine with two different complexes of phytochelatin determines the shapes of the predicted titration curves.

As can be seen in Fig. 6.14, the measured $[CuB_2]$ is significantly lower than that predicted by MINEQL⁺ for both values of $[Cu(I)]_T$ once the excess Cu(I) has been titrated. The Cu(I) titration data suggest less competition than is observed in the bathocuproine titrations. To determine whether the bathocuproine titration curves are consistent with each other, they may be compared with the predicted curves by subtracting the contribution of CuB_2 formed by titration of the excess Cu(I) with bathocuproine (Fig. 6.15). Although the experimental curves lie below the predicted ones, the data from the two ex-

perimental conditions (i.e., two values of $[\text{Cu}]_T$) nearly overlies each other consistent with the model prediction.

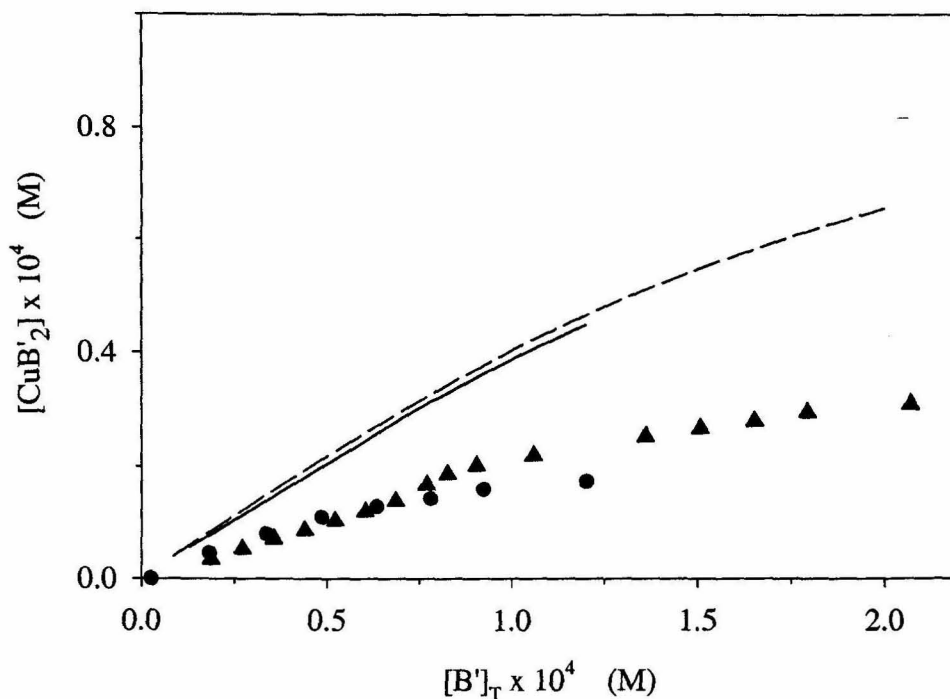


Figure 6.15. Cu(I) speciation in bathocuproine (B) titrations of Cu(I) and phytochelatin (P).

Experimental data:

(●) $[\text{P}]_T = 1.0 \times 10^{-4} \text{ M}$, $[\text{Cu}]_T = 1.4 \times 10^{-4} \text{ M}$.

(▲) $[\text{P}]_T = [\text{Cu}]_T = 1.0 \times 10^{-4} \text{ M}$.

$[\text{B}']_T = [\text{B}]_T$ for (▲) and $[\text{B}']_T = [\text{B}]_T - 8 \times 10^{-5} \text{ M}$ for (●).

$[\text{CuB}'_2] = [\text{CuB}_2]_T$ for (▲) and $[\text{CuB}'_2] = [\text{CuB}_2]_T - 4 \times 10^{-5} \text{ M}$ for (●).

Model predictions (MINEQL⁺):

(—) $[\text{P}]_T = 1.0 \times 10^{-4} \text{ M}$, $[\text{Cu}]_T = 1.4 \times 10^{-4} \text{ M}$.

(- - -) $[\text{P}]_T = [\text{Cu}]_T = 1.0 \times 10^{-4} \text{ M}$.

pH = 6.5, $I = 0.05 \text{ M}$.

As a result of the observed discrepancy, the FITEQL modeling of the bathocuproine titrations gives significantly higher values of the conditional stability constants for

CuP ($10^{16.3}$ vs. $10^{13.8}$) and CuP₂ ($10^{21.2}$ vs. $10^{18.5}$) than those obtained in Cu(I) titrations. The discrepancy in the bathocuproine titrations might result from the slow kinetics of the reactions between the copper complexes of phytochelatin and added bathocuproine.

6.5.3.4 *Kinetic studies of Cu(I)/P/B system*

A possible explanation for the discrepancy observed in bathocuproine titrations could be that the system was not at equilibrium. Preliminary experiments conducted with copper-glutathione system indicated that, in this case, constant absorbance (i.e., stable for > 1 hour) was reached almost immediately after the addition of reactants for both copper and bathocuproine titrations (see section 6.4.3). To test whether equilibrium was reached in a solution containing $[Cu]_T = 1.4 \times 10^{-4}$ M and $[P]_T = 1.0 \times 10^{-4}$ M the absorbance was measured over time after the last addition of bathocuproine to a final concentration $[B]_T = 2.0 \times 10^{-4}$ M (Fig. 6.16). The absorbance increased rapidly for the first few hours and slowly for the next seven days. As seen in Fig. 6.17, the $[CuB_2]$ calculated based on absorbance measurements after the first 600 min (the initial rapid increase of absorbance) is in agreement with the value predicted using the conditional stability constants obtained in Cu(I) titrations. This test suggests that, given longer equilibration time, the bathocuproine titrations would agree with the Cu(I) titrations. However, for such long time periods, it is hard to isolate the chemical processes of oxidation/reduction of copper and phytochelatin from the approach to the equilibrium.

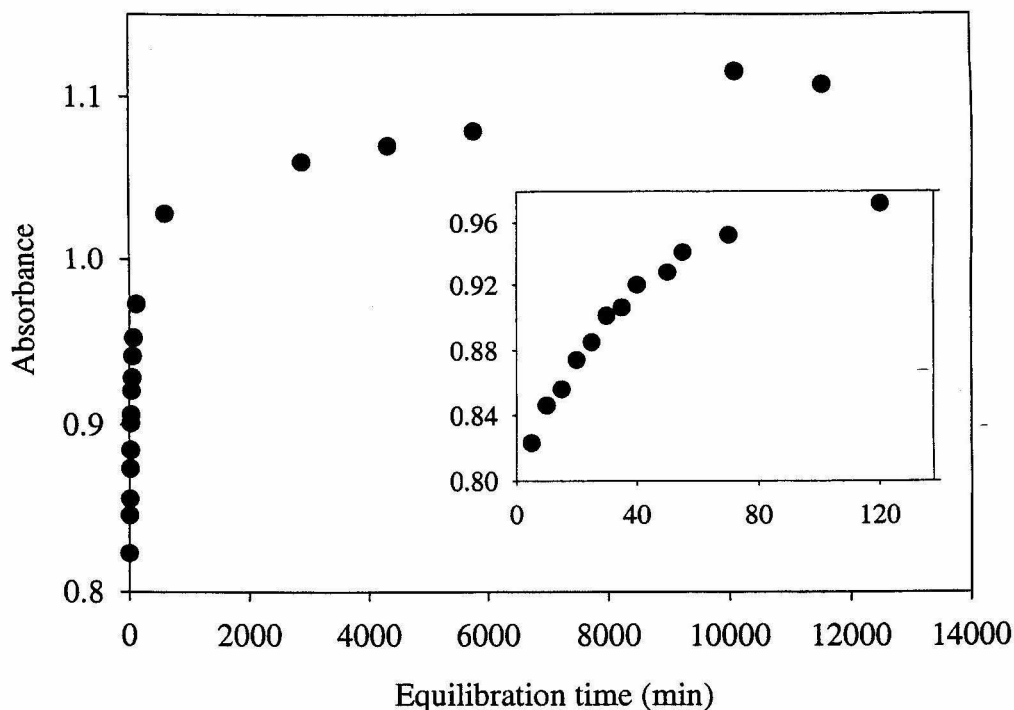


Figure 6.16. Dependence of absorbance measured at 480 nm on equilibration time. $[P]_T = 1.0 \times 10^{-4}$ M, $[Cu]_T = 1.4 \times 10^{-4}$ M, $[B]_T = 2.0 \times 10^{-4}$ M. The sample was prepared by single additions of phytochelatin (P) and Cu(I) followed by the incremental additions ($10 \mu\text{L}$ of 2.0×10^{-3} M solution) of bathocuproine (B) up to its final concentration of 2.0×10^{-4} M. Equilibration time between the incremental additions was 10 min; time $t = 0$ on the graph corresponds to the time of the last addition of B. $\text{pH} = 6.5$, $I = 0.05$ M.

Traces of oxygen could cause changes in the solution composition during long equilibration times. Cu(II), formed by reaction of Cu(I) with O_2 , could be reduced by phytochelatin to give Cu(I) and the disulfide. Phytochelatin could also be oxidized by O_2 with the release of Cu(I). In either case, Cu(I) would then be available to react with bathocuproine (see section 6.5.1.1). This may account for the slow increase of absorbance observed over the seven-day equilibration.

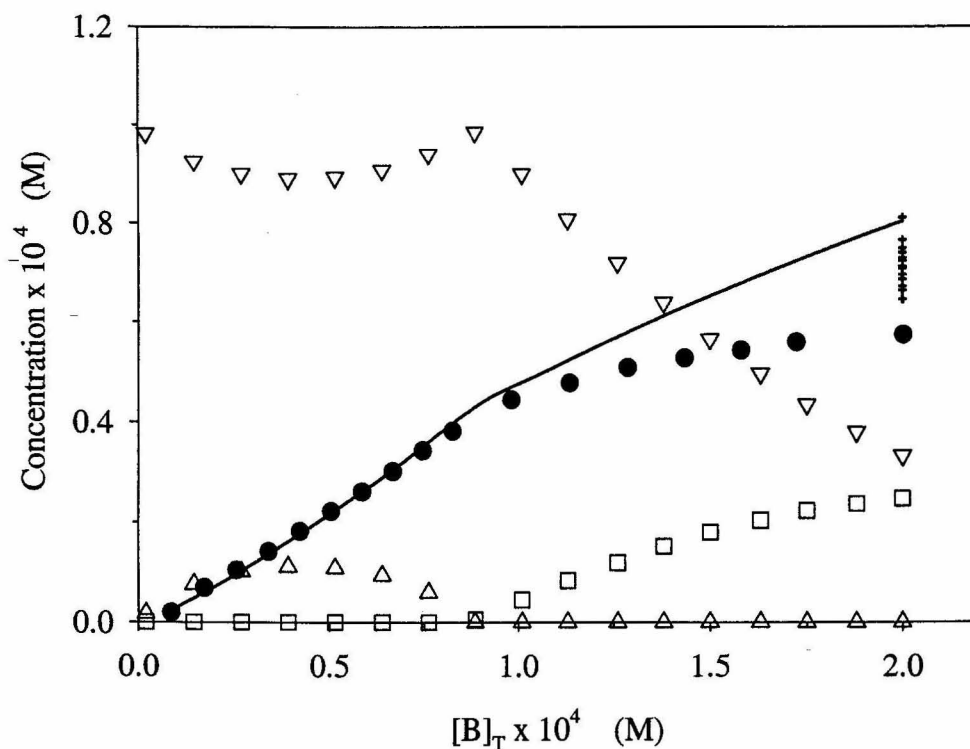


Figure 6.17. Cu(I) speciation in bathocuproine (B) titrations of Cu(I) and phytochelatin (P) based on the conditional stability constants obtained in Cu(I) titration experiments.

$[P]_T = 1.0 \times 10^{-4} \text{ M}$, $[Cu]_T = 1.4 \times 10^{-4} \text{ M}$.

pH = 6.5, $I = 0.05 \text{ M}$.

Experimental data: (●) $[CuB_2]$ calculated from measured absorbance at 480 nm.

(+) Change of $[CuB_2]$ during the first 600 min for the last titration point.

Model predictions (MINEQL⁺):

(—) $[CuB_2]$, (Δ) $[BCu_2P]$, (▽) $[CuP]$, (□) $[CuP_2]$.

For the values of the constants see Table 6.9.

At this point it is not clear why such long equilibration times would be necessary when only Cu(I), phytochelatin and bathocuproine are present in the solution.

Similar discrepancies between experiments which varied in the manner of analyte addition were observed in bathocuproine titrations of glutathione (see section 6.5.2) and

previous experiments with Cu(I) phytochelatin complexes (Mehra and Mulchandani, 1995; Mehra and Winge, 1988). In the latter work, different complex stoichiometries were obtained in the case of incremental and one time additions of Cu(I) to the solution of phytochelatin isolated from yeast. Formation of different species in different experimental protocols can not be excluded.

The discrepancy of bathocuproine titration experiments requires the further investigation.

6.6 Implications of Cu-phytochelatin complexation studies

The conditional stability constants obtained in this study indicate that phytochelatin forms very strong with Cu(I). Even higher conditional stability constants may be expected at higher pH values and for longer-chain phytochelatins. If present in natural waters, such complexes could influence copper speciation.

The study of Cu(I) – phytochelatin interactions presented here was performed under low oxygen concentrations consistent with intracellular conditions. The importance of such complexes for copper complexation in oxic waters is questionable since both Cu(I) and phytochelatin can be expected to be oxidized. However, both Cu(I) and thiols have been reported to be present in the ocean at detectable concentrations (Moffett and Zika, 1988; le Gall and van den Berg, 1997). The balance between the oxidation-reduction and production or loss of Cu(I), phytochelatin and their complexes would determine their ambient concentrations and their effect on copper speciation.

6.6.1 Sources, occurrence, and inertness of Cu(I) in natural waters

It has been suggested that Cu(I) is easily oxidized in seawater. In laboratory studies of Cu(I) oxidation in seawater in the range of Cu(I) concentrations of ca. 0.1 to 5 μM , Cu(I) has been shown to have a half life of only 4 to 6 minutes (Moffett and Zika, 1983). Extrapolation of laboratory data for Cu(I) oxidation rates to natural levels was based on the assumption that Cu(I) was stabilized by chloride, which forms strong complexes with Cu(I) and is present at high concentrations in seawater.

However, Cu(I) has been measured in surface seawater at subnanomolar concentrations and have been found to persist even in filter sterilized samples (Moffett and Zika, 1988; Moffett and Zika, 1987a).

There are many photochemical and biological reactions that result in redox cycling of Cu(II) and Cu(I) and can maintain significant steady-state Cu(I) concentrations (Fig. 6.18). It has been shown that photooxidation of dissolved organic matter leads to the generation of hydrated electrons which react rapidly to form redox active agents such as organic free radicals, O_2^- and H_2O_2 (Zafirou, 1977; Zika, 1981). Both O_2^- and H_2O_2 have been shown to reduce Cu(II) to Cu(I) under the seawater conditions (Davies et al., 1976; Klug-Roth and Rabani, 1976; Moffett and Zika, 1983). There is also evidence for metal reduction mediated by dissolved organic matter (DOM) via complexation with such functional groups of DOM as quinones or by electron shuttling between the metal and cells through the DOM (Sunda et al., 1983; Waite and Morel, 1984; Choppin et al., 1985; Royer et al., 2000; Gu et al., 2000).

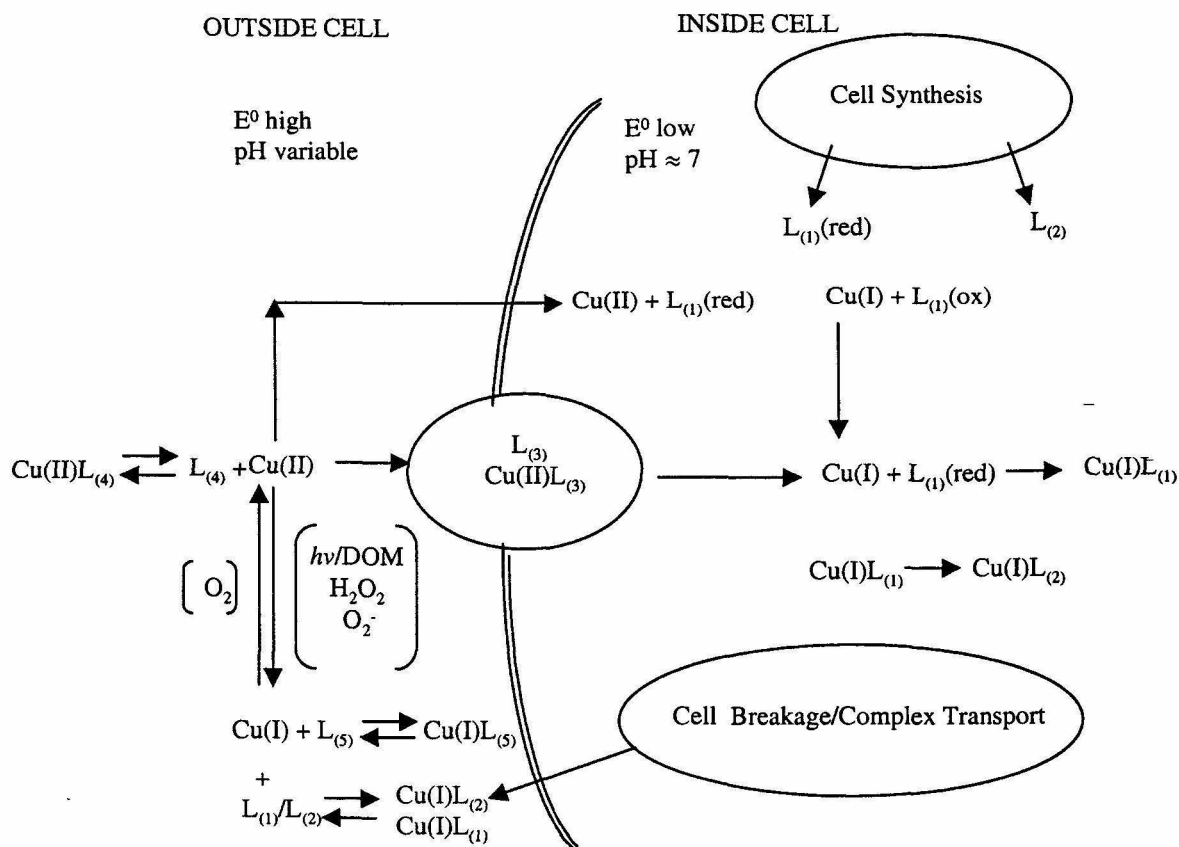


Figure 6.18. A simplified model for redox cycling of copper ions in natural waters. Ligands $L_{(1)}$, $L_{(2)}$, $L_{(3)}$ are synthesized by a cell; ligands $L_{(4)}$ and $L_{(5)}$ represent a variety of extracellular Cu(II) and Cu(I) ligands respectively. Intracellular ligand $L_{(1)}$ represents glutathione, that can reduce Cu(II) and bind Cu(I) . The complex $\text{Cu(I)}L_{(1)}$ can then transfer reduced Cu(I) to other proteins, peptides and enzymes (ligand $L_{(2)}$), e.g., metallothionein and phytochelatins. Cu(II) is thermodynamically stable under oxic conditions, while Cu(I) is formed as a result of Cu(II) reduction by redox active species (e.g., O_2^- and H_2O_2) present in water or through biological reduction. Biological reduction can take place either inside a cell (interaction with reduced form of $L_{(1)}$, $L_{(1)}(\text{red})$) or in a cell membrane (interaction with $L_{(3)}$). Cu(I) in oxic waters can be stabilized by a complexation with an extracellular Cu(I) ligand $L_{(5)}$ (e.g., Cl^-) or ligands synthesized by a cell $L_{(1)}(\text{red})$ and $L_{(2)}$ (e.g., glutathione and phytochelatins).

Biological reduction of Cu(II) can take place inside a cell via biologically produced reductants such as glutathione (Kasower, 1976), or in the cell membrane (Jones et al., 1987; Hassett and Kodman, 1995).

Reducing agents such as glutathione are normally present in cells at high concentrations (ca. mM), which can be increased with copper uptake (Rama and Prasad, 1998). It has been proposed that copper entering the cell is initially complexed by glutathione. Cu(II) is reduced by glutathione to Cu(I) and a stable Cu(I)-glutathione complex is formed (note that a similar reduction of Cu(II) by phytochelatin has been demonstrated in this study (see section 6.5)). After the Cu(I)-glutathione complex is formed, the metal is transferred to other proteins and enzymes such as superoxide dismutase, metallothionein and phytochelatin (Cirilio et al., 1990; Bae and Mehra, 1997).

Cu(II) can also be reduced during transport through the cell membrane. Such reduction has been observed in phytoplankton (Jones et al., 1987) and in yeast (Hassett and Kodman, 1995). Studies of Cu(II) uptake by phytoplankton in which $[Cu^{2+}]$ was controlled by EDTA have indicated that Cu uptake rates exceed the maximum supply of non-complexed copper species to the surface of three species of phytoplankton by factors of 13 to 30 (Sunda and Huntsman, 1995). These uptake rates could be sustained if the algae reduced Cu(II)EDTA to Cu(I) at their surfaces.

Reported levels of Cu(I) concentrations in natural waters are on the order of 5-10% of the total copper concentration (Moffett and Zika, 1988). Concentrations of Cu(I) are commonly determined with dimethyl-phenanthroline, a highly specific and sensitive Cu(I)-binding ligand or with its more soluble derivative, bathocuproine disulfonic acid, which was also used in this study.

6.6.1.1 Measurements of Cu(I) concentration with 2,9-dimethyl-1,10-phenanthroline (dmp)

Cu(I) concentrations at subnanomolar levels were measured in seawater using dmp (Moffett and Zika, 1988). The Cu(I)-dmp complex was pre-concentrated by solvent extraction with methylene chloride and back extraction into dilute acid, followed by atomic absorption analysis for total extractable copper. Interference of Cu(II) in the presence of reducing agents was inhibited using ethylenediamine as a masking ligand.

It was found that the signal of Cu(I) increased slowly between the reagent addition and the extraction. This slow increase in the signal could be caused by a slow interference process or a slow exchange of Cu(I) between a very strong complex and dmp. Since distinguishing between the two processes was not possible, the extraction was always carried out immediately after the reagent addition. The authors recognized that a significant, relatively nonlabile pool of Cu(I) could have been neglected.

The present study has demonstrated that very strong Cu(I) complexes are formed with ligands like phytochelatin. Experiments in which mixtures of Cu(I) and phytochelatin were titrated with bathocuproine suggest that it can take more than two hours to reach the equilibrium predicted by titrations with either Cu(I) or phytochelatin (see section 6.5.3.5). If similar Cu(I) complexes were present in natural samples, they would not be measured in dmp assay leading to underestimation of Cu(I) concentrations.

When strong Cu(I) complexes are present, the kinetic data for oxidation based solely on inorganic Cu complexation are inapplicable since even nanomolar concentrations of S donor ligands can dominate Cu(I) speciation at low levels of copper concentra-

tions (Moffett and Zika, 1988; Rozan and Benoit, 1999). The occurrence of strong Cu(I) complexes in the water column is plausible. If such complexes are present, then it is likely that both the concentration and life-time of Cu(I) in natural waters has been underestimated.

6.6.2 Thiols as strong Cu(I) binding ligands in natural waters

Strong Cu(I)-binding ligands, such as glutathione and phytochelatin, are produced by various phytoplankton species. These organic compounds can be released into natural waters as a result of sloppy feeding, breakdown of dead organisms or exudation from living microorganisms. Thiols are known to occur in both anoxic and oxic natural waters (Anderson et al., 1988; Matrai and Veter, 1988; Luther III et al., 1991).

Due to analytical difficulties, there are few measurements of specific thiols. Glutathione has been detected in the upper water column at nanomolar concentrations with a maximum concentration (ca. 10-20 nM) near the fluorescence maximum and subnanomolar levels in deeper waters (Le Gall and van den Berg, 1998). The voltammetric measurements do not distinguish between the reduced and oxidized forms of glutathione so the actual concentration of strong Cu(I)-binding ligand, reduced glutathione, would be lower than the total reported glutathione concentration (Le Gall and van den Berg, 1993). It is possible that the total thiol concentration reported based on glutathione calibration also includes phytochelatins. However, a study of one type of phytochelatin has shown that it produces a peak at more negative potential than glutathione (Scarano and Morelli,

1996). Thus, the phytochelatins are likely to give a distinct response that does not coincide with that of glutathione in voltammetric measurements.

Lower concentrations of phytochelatin than glutathione can be expected in natural waters since glutathione is usually present in cells whereas phytochelatin production is induced only at elevated metal concentrations and is terminated when the metal is complexed. However, the stability of Cu(I) complexes with phytochelatin is orders of magnitude higher than that of glutathione complexes so Cu(I) complexation with phytochelatin can be important even at lower concentrations of this ligand.

Thiols, such as cysteine and glutathione, are known to be easily oxidized with the formation of a disulfide bridge. The smaller cysteine molecules are oxidized more readily than glutathione and the oxidation rates for larger molecules of phytochelatin have not been reported. Since the thiol-containing compounds are readily exuded and taken up by the natural community of microorganisms (Suttle et al., 1991), the dissolved thiol concentrations in a water column are a balance between in situ production, uptake and degradation processes.

6.6.3 Cu(I)-thiol complexation in natural waters.

Formation of Cu(I)-thiol complexes can occur through: 1) redox process of thiol oxidation and Cu(II) reduction to Cu(I) followed by Cu(I) reaction with the excess of the reduced thiol; 2) direct reaction of Cu(I) with thiols in natural waters and; 3) reaction of Cu(I) with thiols inside the cells followed by release of the complex from the cells. Under intracellular (reducing) conditions, copper is likely to be present as Cu(I) and the

concentrations of thiol-containing ligand are high; thus, complexation is likely to occur inside the cells. Such complexes then can be released into the water column.

Complexation of Cu(I) with thiol-containing ligands would stabilize both Cu(I) and thiols in oxic natural waters. Similar stabilization of sulfides in metal complexes has been observed in oxic river waters (Rozan et al., 1999). Such Cu(I) complexation could explain the observed persistence of Cu(I) at night in the absence of active reducing agents and the slow increase in Cu(I)-dmp signal (Moffett and Zika, 1988).

It has been shown that when seawater amended with well-defined ligands is titrated with Cu(II) identical responses are obtained for ligands that have a greater affinity for Cu(I) (i.e., glutathione and cysteine) and for those reported to control Cu(II) speciation in natural waters (Leal and van den Berg, 1998). Thus it is possible that at least some of the ligands that are assumed (based on Cu(II) titrations) to bind Cu(II) are in fact Cu(I) binding ligands which stabilize the +I oxidation state in seawater.

Polarographic studies of copper complexation with ligands produced by three copper-stressed phytoplankton species in seawater have shown formation of several distinct and very strong complexes. The stability constants determined for the ligands were very high ($\log K = 22.6 - 39.1$) relative to model ligands in the literature (Croot et al., 1999). One of the suggested explanations for such large constants was formation of Cu(I) rather than Cu(II) complexes. For Cu(I) complexes calculated logarithms of stability constants would be smaller by a factor of two (based on a one-electron rather than two-electron transfer reaction). In case of Cu(I) complexation, it is likely that these biologically-produced ligands are S donors, containing thiol groups. The conditional stability constants determined in the present study for Cu(I)-complexes with the synthetic

phytochelatin can not be directly compared to the constants reported by Croot et al. (1999), which are independent of pH. Nevertheless, since phytochelatin is produced by many species of phytoplankton, it could be one of the strong copper-binding ligands studied by Croot et al. (1999).

Thus the +I oxidation state of Cu may be important under natural conditions and Cu(I) may be strongly complexed and thus stabilized by thiol-containing, biologically-produced ligands such as glutathione and phytochelatins. In turn, these ligands may be at least partially responsible for strong Cu binding in natural waters.

6.7 Concluding remarks

Investigation of Cu(II) interactions with phytochelatin using an EPR technique and a bathocuproine assay indicated that Cu(II) is not stable in presence of phytochelatin. Titration experiments with Cu(I), bathocuproine and phytochelatin have shown that Cu(I) forms complexes of high stability with phytochelatin. Formation of Cu(I)-phytochelatin complexes was studied using a spectroscopic technique in which bathocuproine is added as a competing ligand and the formation of the 1:2 copper-bathocuproine complex is followed spectrophotometrically.

The method was validated in experiments with glutathione, which is structurally similar to phytochelatin and forms Cu(I) complexes of known stability. Conditional stability constants of Cu(I) complexes with glutathione were obtained in Cu(I) titrations of glutathione and bathocuproine and in bathocuproine titrations of Cu(I) and glutathione.

The results of Cu(I) titrations of glutathione/bathocuproine for the two different concentrations of bathocuproine agreed well. A stronger conditional stability constant was obtained for the CuG complex in bathocuproine titrations of Cu(I)/glutathione than that obtained in Cu(I) titrations of bathocuproine/glutathione.

To account for the experimental observations an additional ternary complex BCuG was introduced into the modeling of the titration data. Analysis of the potential interference of the ternary complex with spectroscopic measurements of the 1:2 Cu(I)-bathocuproine complex indicated that such interference was unlikely. The experiments with glutathione illustrated the applicability of the spectroscopic technique with bathocuproine for determination of conditional stability constants of Cu(I) complexes with thiol-containing ligands.

Three series of titrations were performed to obtain conditional stability constants of Cu(I) complexes with phytochelatins: titrations of phytochelatins/bathocuproine with Cu(I), titrations of Cu(I)/bathocuproine with phytochelatins and titrations of phytochelatins/Cu(I) with bathocuproine. The experimental data obtained in Cu(I) titrations of phytochelatins/bathocuproine and in phytochelatins titrations of Cu(I)/bathocuproine can be explained by formation of Cu(I)-phytochelatins complexes of 1:1 and 1:2 stoichiometries. In addition to these complexes, a mixed polynuclear complex (BCu₂P) appears to be present in the system. Introduction of this complex into the modeling of the titration experiments accounts for the experimental observations. The following conditional stability constant values were obtained in Cu(I) and phytochelatins titrations:

$$\log K_{CuP}^{cond} = 13.8 \pm 0.3, \log \beta_{CuP_2}^{cond} = 18.3 \pm 0.3, \log \beta_{BCu_2P}^{cond} = 27.8 \pm 0.3.$$

Conditional stability constants obtained in bathocuproine titrations are several orders of magnitude higher than those obtained in Cu(I) and phytochelatin titrations: $\log K_{CuP}^{cond} = 16.3$ and $\log \beta_{CuP_2}^{cond} = 21.2$. This discrepancy suggests that different species may form in the solution depending on the manner of analyte additions and/or long pre-equilibration times may be necessary to reach equilibrium. With long equilibration times, however, it is difficult to distinguish between oxidation-reduction of Cu(II) and/or the thiol and the approach to equilibrium. At this point it is not clear why pre-equilibration times should be so different depending on the order of analyte addition.

The sensitivity of the spectroscopic method does not permit experiments to be performed at very low copper concentrations that would be relevant to natural waters. Formation of different species with different molar ratios of copper and phytochelatin is possible under such conditions.

Finally, since a synthetic phytochelatin was used in this study, the results should be considered as a first approximation to the characterization of native copper-phytochelatin complexes. It has been suggested that synthetic peptides and phytochelatins isolated from cells that contain the same number of glutathione units should have similar metal binding properties (Bae and Mehra, 1997). However, some differences may arise from the different structural arrangement of the natural and synthetic compounds. Certainly different metal-binding properties would be expected for the longer chain ($n > 2$) phytochelatins.

APPENDIX 6A

STUDIES OF Cu(II)-PHYTOCHELATIN INTERACTIONS USING COUPLED SEC-ICP/MS TECHNIQUE

Phytochelatins isolated from *Silene vulgaris* and substituted with Cu(II) ions has been shown to form complexes that can be observed with coupled SEC/ICP-MS technique (Leopold and Günther, 1997). Since the technique is applicable for determination of conditional stability constants of strong metal complexes (see Chapter 3), its applicability for studies of Cu(II)-phytochelatins complexation was examined.

Experimental methods

Reagents

The chromatographic elution solutions (10 mM ammonium acetate) were prepared by titrating acetic acid (99.7 vol. %; Seastar) with ammonium hydroxide (21.7 vol. %; Seastar) to pH = 7.0 and diluting with ultrapure water (18 M Ω cm, Millipore Milli-Q system). Stock solutions and standards were prepared gravimetrically in the elution solution from 1.00 g L⁻¹ Cu(II) reference solutions (Fisher Scientific), ethylenediaminebis(2-hydroxyphenyl) acetic acid (EDDHA, 97%, Aldrich), 2-mercaptoethanol (ME, Sigma)

or synthetic phytochelatins (P, Caltech Biopolymer Synthesis Center). The standard tuning solution for ICP-MS, containing 0.50 mg/L of Li, Y, Tl, was prepared from 1.00 mg L⁻¹ solutions of these elements (ULTRA Scientific). The internal standard solution was prepared from a 10.00 g L⁻¹ Ga standard solution (ULTRA Scientific) by dilution in 25% (vol. %) nitric acid (Seastar, Canada).

Instrumentation

For the description of a coupled SEC-ICP/MS system, column specification and ICP/MS operating parameters, see Chapter 3.

Sample preparation

To minimize phytochelatins oxidation, sample preparation was done on a laboratory bench under a flow of nitrogen. The Cu-phytochelatins complex was prepared by addition of Cu(II) up to the final concentration of 1.0 µM to the solution of phytochelatins ([P] = 5.0 µM) and 2-mercaptoethanol ([ME] = 500 µM). After measurements of Cu recovery on the column, solutions of Cu-phytochelatins complex with EDDHA as a competing ligand were prepared by addition of EDDHA up to three different final concentrations: 0.25, 0.5 and 1.0 µM to the solution of Cu-phytochelatins ([Cu]_T = 1.0 µM, [P]_T = 5.0 µM).

Results and discussion

Solutions containing the Cu-phytochelatin complex were injected into the SEC, and Cu concentrations in the eluant were measured by ICP/MS; only about 50% of the initially added Cu was recovered from the column (Fig. 1A, samples # 1 and 2). Since the initial solution contained a five-fold excess of phytochelatin over Cu, it was expected that all Cu should be present in a complex with phytochelatin. However, such low Cu recoveries indicate that either Cu was only partially complexed or that the complex is unstable under the experimental conditions. The complex instability can be caused by oxidation/reduction reactions of Cu(II) and phytochelatin, by complex dissociation under non-equilibrium conditions in the chromatographic column or by a combination of these processes. Based on the results of SEC/ICP-MS measurements, it is difficult to distinguish between oxidation/reduction and non-equilibrium dissociation processes.

After the observation of partial Cu recovery from the column, the Cu-phytochelatin sample was split into four sub-samples. Different amounts of EDDHA were added to three of these sub-samples. The resulting four sub-samples (three with added EDDHA and one without EDDHA) were injected into the column and Cu concentration in CuP peak eluted from the column was measured with ICP-MS (Fig. 1A, samples # 3, 4 and 5). The Cu recovery decreased in the Cu-phytochelatin sample without EDDHA. The same concentration of Cu was measured in Cu-P peak in three samples with added EDDHA as in the absence of EDDHA indicating that EDDHA was not competing for copper ions.

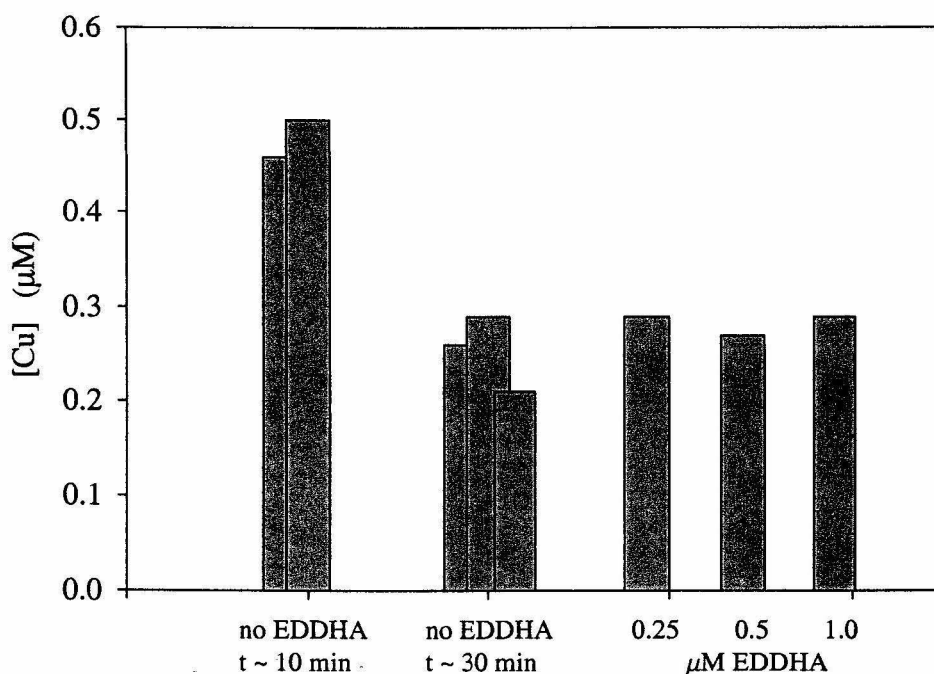


Figure 6A.1. Cu concentration measured in Cu-phytochelatin peak with ICP-MS after the complex elution from the SEC column.

Conditions: $[P]_T = 5.0 \mu\text{M}$, $[Cu]_T = 1.0 \mu\text{M}$; pH = 7, $I = 10 \text{ mM}$ (ammonium acetate). The groups of bars correspond to replicates analyzed within 10 min of solution preparation (first group) and within 30 min of solution preparation (second group). The last three bars correspond to the samples with three different concentrations of added EDDHA. These samples were analyzed within 10 min of EDDHA addition and within 30 min of Cu and phytochelatin addition.

If the complex were dissociating under the non-equilibrium conditions on the column, it could be expected that Cu recovery would be the same in the later injections of the same sample (or perhaps higher due to the column contamination with Cu lost from

the complex during the first two injections). The fact that Cu recovery decreased over time is indicative of redox reactions in the solution.

The absence of any competition from the added EDDHA can be the result of very strong Cu(II) complexation by phytochelatin or reduction of Cu(II) to Cu(I), which is likely to form much weaker complexes with EDDHA than Cu(II). Considering the decreased recovery of Cu with time and presence of the reducing agent mercaptoethanol, it is more likely that Cu(II) is reduced than that phytochelatin outcompetes EDDHA for Cu(II). Thus the Cu eluting from the column with phytochelatin is probably Cu(I) rather than the Cu(II) originally added Cu(II).

APPENDIX 6B

EXPERIMENTAL TITRATION DATA NOT CONSIDERED IN THE OPTIMIZATION OF CONDITIONAL STABILITY CONSTANTS

The table includes experiments that produced inconsistent results (bathocuproine titrations) and experiments that were performed with a lower quality bathocuproine (Cu(I) titrations).

Experimental conditions (pH = 6.5, $I = 0.05$ M)	[Titrant] x 10^4 M	Absorbance, 480 nm	Dilution
Bathocuproine (B) titrations of glutathione (G) and Cu(I) [G] _T = 4.0×10^{-4} M [Cu] _T = 0.24×10^{-4} M [B] _T = $0.01 - 0.90 \times 10^{-4}$ M	0.01	0.032	0.995
	0.02	0.035	0.909
	0.03	0.039	0.985
	0.04	0.043	0.980
	0.05	0.046	0.976
	0.14	0.092	0.971
	0.23	0.135	0.966
	0.31	0.177	0.962
	0.40	0.172	0.957
	0.48	0.181	0.952
	0.57	0.196	0.948
	0.65	0.201	0.943
	0.74	0.212	0.939
	0.82	0.237	0.934
	0.90	0.234	0.930

Experimental conditions	[Titrant] x 10 ⁴ M	Absorbance, 480 nm	Dilution
Cu(I) titrations of glutathione (G) and bathocuproine (B) [B] _T = 2.0 x 10 ⁻⁴ M [G] _T = 4.0 x 10 ⁻⁴ M [Cu] _T = 0.12 - 2.75 x 10 ⁻⁴ M	0.12	0.169	0.995
	0.25	0.321	0.909
	0.37	0.439	0.985
	0.49	0.390	0.980
	0.60	0.336	0.976
	0.72	0.298	0.971
	0.84	0.276	0.966
	0.95	0.257	0.962
	1.07	0.246	0.957
	1.18	0.233	0.952
	1.29	0.223	0.948
	1.40	0.214	0.943
	1.51	0.208	0.939
	1.62	0.197	0.934
	1.73	0.188	0.930
	1.83	0.180	0.926
	1.94	0.172	0.922
	2.05	0.165	0.917
	2.15	0.157	0.913
	2.25	0.149	0.909
	2.35	0.142	0.897
	2.46	0.133	0.893
	2.56	0.126	0.889
	2.65	0.122	0.885
	2.75	0.118	0.881

Experimental conditions	[Titrant] x 10 ⁴ M	Absorbance, 480 nm	Dilution
Cu(I) titrations of phytochelatins (P) and bathocuproine (B) [B] _T = 1.8 x 10 ⁻⁴ M [P] _T = 1.0 x 10 ⁻⁴ M [Cu] _T = 0.12 - 1.62 x 10 ⁻⁴ M	0.12	0.071	0.995
	0.25	0.148	0.909
	0.37	0.226	0.985
	0.49	0.299	0.980
	0.60	0.369	0.976
	0.72	0.427	0.971
	0.84	0.471	0.966
	0.95	0.506	0.962
	1.07	0.539	0.957
	1.18	0.570	0.952
	1.29	0.609	0.948
	1.40	0.668	0.943
	1.51	0.690	0.939
	1.62	0.660	0.934
[B] _T = 1.4 x 10 ⁻⁴ M [P] _T = 1.0 x 10 ⁻⁴ M [Cu] _T = 0.12 - 1.62 x 10 ⁻⁴ M	0.12	0.047	0.995
	0.25	0.125	0.909
	0.37	0.199	0.985
	0.49	0.270	0.980
	0.60	0.331	0.976
	0.72	0.381	0.971
	0.84	0.413	0.966
	0.95	0.404	0.962
	1.07	0.436	0.957
	1.18	0.464	0.952
	1.29	0.503	0.948
	1.40	0.534	0.943
	1.51	0.502	0.939
	1.62	0.473	0.934

Experimental conditions	[Titrant] x 10 ⁴ M	Absorbance, 480 nm	Dilution
Cu(I) titrations of phytochelatin (P) and bathocuproine (B) [B] _T = 0.7 x 10 ⁻⁴ M [P] _T = 1.0 x 10 ⁻⁴ M [Cu] _T = 0.37 - 1.62 x 10 ⁻⁴ M	0.37	0.073	0.909
	0.49	0.123	0.985
	0.60	0.157	0.980
	0.72	0.175	0.976
	0.84	0.186	0.971
	0.95	0.197	0.966
	1.07	0.205	0.962
	1.18	0.226	0.957
	1.29	0.247	0.952
	1.40	0.270	0.948
	1.51	0.245	0.943
	1.62	0.225	0.939

Chapter 7

SUMMARY AND CONCLUSIONS

In this work some questions concerning metal speciation in natural waters have been addressed. Metal competition between organic ligands was examined by coupling a chromatographic technique for the separation of metal-organic complexes with a metal-specific detection technique. Limitations of chromatographic methods for equilibrium metal speciation studies due to the non-equilibrium conditions during species separation were investigated both experimentally and theoretically. Copper complexation by potentially important metal-binding ligands have been studied. The developed method was applied to studies of copper interactions with humic acids. Copper interactions with a synthetic phytochelatin (used as a model for natural phytochelatins, a class of thiol-containing biogenic oligopeptides) were investigated using competitive ligand equilibration coupled with spectroscopic detection. The purpose of this chapter is to provide a summary of the findings of each section of the study.

7.1 Direct quantification of metal-organic interactions by size-exclusion chromatography (SEC) and inductively coupled plasma mass spectrometry (ICP-MS)

Isolation of strong metal-binding ligands present in natural waters at nanomolar concentrations for their structural characterization has not been successful so far. Information on the nature of the strong metal complexes can be inferred from their behavior in different fractionation schemes and through quantitative information on metal-organic interactions. However, the fractionation (primarily by chromatographic methods) is rarely used for metal titration experiments that provide quantitative information on metal complexation. Such information as the complexation capacity and metal affinity of naturally-occurring ligands can be incorporated into biogeochemical modeling to predict equilibrium metal speciation in natural water systems. Limitations of chromatographic techniques for equilibrium metal speciation studies due to the violation of equilibrium conditions during the separation and competition between trace metals for naturally-occurring ligands also need further investigation.

The method developed in this study allows direct quantification of strong metal-organic interactions through determination of conditional stability constants of metal complexes with strong organic ligands in competitive equilibration experiments. In this method, SEC was directly coupled with ICP-MS.

Information on the nature of strong metal-binding ligands can be inferred from the complex size as determined by size-exclusion chromatography. Molecular size-

calibration of the column was performed with polystyrene-sulfonate standards and the effects of hydrophobic interactions on the column were determined.

Metal concentrations in different organic fractions were measured directly in separated fractions with ICP-MS which minimized sample dilution. Method validation was performed with well-defined organic ligands in ligand competition studies with a single metal and a binary metal mixture. These experiments have demonstrated a good agreement between experimental and predicted from equilibrium calculations metal speciations in both types of experiments and between known and experimentally determined values of conditional stability constants for a chosen metal complex. However, experiments with ligands forming less stable and/or more labile complexes have demonstrated the limitations on the applicability of this method and on previous interpretations of similar chromatographic techniques. During the chromatographic separation the (original) equilibrium species distribution is maintained only for the kinetically-inert species. For metal complexes that are partially labile under the chromatographic conditions, the metal is redistributed among various chromatographic fractions or lost to the column.

The method sensitivity does not permit its application to unpolluted natural samples where trace metals and strong ligands of interest are present at nanomolar concentrations. However, it could be applied to studies of contaminated waters with elevated metal concentrations or to pre-concentrated natural water samples.

Mathematical modeling was performed to gain a better understanding of non-equilibrium dynamic processes occurring during chromatography and to determine extent to which the method can be applied to complexes that are partially labile under the experimental conditions.

7.2 Mathematical modeling of the chromatographic behavior of metal complexes

The main concerns of chromatography have been isolation of an analyte of interest from mixtures or sample pre-concentration. Although limitations on the applicability of chromatographic techniques to metal speciation studies have been recognized, a mathematical description of the alteration of equilibrium speciation during chromatographic separation has not been developed.

The main goal of this study was to develop a mathematical model that would account for a sample alteration during chromatographic separation. This would allow estimation of the equilibrium metal speciation for metal complexes that are partially labile under the experimental conditions. The mathematical model was developed for a coupled SEC/ICP-MS technique.

Since both the complexed and "free" metals are measured by ICP-MS, the developed model describes the behavior of these species on the chromatographic column. [Note that the "free" metal is taken to include all forms of metal other than the complex(es) initially present in the sample injected onto the column.] The model takes into account several processes which occur during the chromatographic separation: dissociation of the metal complex, diffusion and the "free" metal adsorption onto the column.

The modeling has demonstrated that there are several limiting regimes for the chromatographic behavior of metal complexes. Two essential regimes are distinguished by the time scale of diffusion relative to that of complex dissociation: a diffusion-

dominated and an advection-dominated regime. In the advection-dominated regime, effects of dissociation on the location of the peak of the complex in the elution profile are negligible and diffusion changes only the shape of the profile. A significant amount of metal may be lost from the complex on the experimental time scale due to the dissociation, but the elution times/volumes of peak maxima are determined by the effective velocities of the analytes. In the diffusion-dominated regime, advection is slow compared to the effects of diffusion and dissociation; the complex is transported mainly via diffusion, while dissociating. The advection-dominated regime was operative for all the experiments in the present study.

For this regime the effective velocities of the analytes can be determined from the peak positions in the elution profiles. The advection-dominated regime is further divided based on the extent of complex dissociation on the experimental time scale. If only a small amount of the complex dissociates on the experimental time scale, then the complex is called "quasi-inert". When most of the complex dissociates during the chromatographic separation, the complex is called "labile".

The system under study is described by mass conservation equations for complexed and "free" metal concentrations. The exact solution is obtained for the concentration of the complexed metal and an approximate solution is obtained for the equation for the "free" metal. Further approximations corresponding to the diffusion- and advection-dominated regimes are made to use the equations describing the system for the interpretation of experimental data.

The results of the mathematical modeling suggest that, for quasi-inert complexes, the (initial) equilibrium concentration of the complex and also, in some cases, the rate

constant for dissociation of the complex can be obtained. This may be done by varying the flow rate and thus the elution time of the complex, plotting the measured metal concentrations against elution time and fitting the experimental data. Parameters of the obtained fit can be used to calculate the rate constant and/or the equilibrium metal complex concentration. In the case of labile complexes, the rate constant for dissociation may be directly estimated from the position of the peak for the "free" metal released from the dissociating complex.

The model is based on necessary simplifying assumptions that lead to some uncertainties in the values determined for dissociation rate constants and the equilibrium metal complex concentrations.

Although developed for the SEC/ICP-MS system, the model is applicable to all chromatographic techniques used for metal complexation studies that involve species separation and violation of equilibrium conditions.

7.3 Studies of copper-humic interactions using SEC/ICP-MS

The goal of this study was to investigate strong copper-humic interactions using SEC/ICP-MS method described in previous chapters. The work was performed with Suwannee River humic acid, which is commonly used as a reference humic acid. The experiments with 50 and 100 mg/L humic acids have demonstrated that copper complexes with humic acids are kinetically labile on the column for copper additions comparable to ambient copper concentrations. All experiments had to be performed at very low total copper concentrations (ca. nanomolar) near the detection limit for ICP-MS and strong

matrix effects were associated with the high humic acid concentrations examined. This resulted in significant uncertainties in quantifying the concentrations of copper associated with humic acids.

The results obtained suggest that strong Cu-binding sites in humic acids are present at very low concentrations. These sites are not available to bind added Cu. The strong sites reported in previous studies were not detected by the SEC/ICP-MS method. Conditional stability constant of copper and humic acids complex appears to be greater than 10^{12} in competitive equilibration experiments with EDDHA (ethylenediiminobis (2-hydroxyphenyl) acetic acid). However, the consistent value of the total ligand concentrations were not obtained for 50 and 100 mg/L humic acids. Since the accurate value of this parameter is necessary for determination of the conditional stability constant, the constant could not be determined from these experiments.

Experiments with humic acids have demonstrated that the method can also be used for isolation of strong metal-binding ligands from a mixture of ligands of different strength. This may be important for natural samples where a mixture of ligands is present and the effects of strong metal-binding ligands on metal speciation are mitigated by more abundant but weaker ligands.

7.4 Investigation of copper-phytochelatin complexation using a spectroscopic technique with bathocuproine

A synthetic phytochelatin was used as a model compound for investigation of copper-binding properties of natural phytochelatins, a class of thiol-containing oligopeptides produced by plants and fungi. Since copper is an effective inducer of phytochelatin production and is suggested to form the strongest complexes with the peptide relative to other metals, the stability of copper-phytochelatin complexes was investigated. Investigation of Cu(II) interactions with phytochelatin using an EPR technique and a bathocuproine assay indicated that Cu(II) is not stable in the presence of phytochelatin. Formation of Cu(I)-phytochelatin complexes was studied using a spectroscopic technique in which bathocuproine is added as a competing ligand and the formation of the 1:2 copper-bathocuproine complex was followed spectrophotometrically.

The method was validated with glutathione, the precursor to phytochelatin biosynthesis. Stability constants for 1:1 and 1:2 Cu(I)-glutathione complexes have been reported. The results of Cu(I) titrations of glutathione and bathocuproine for two different concentrations of bathocuproine agreed well. The results of bathocuproine titrations of glutathione and Cu(I) have shown some discrepancies with Cu(I) titrations. A ternary glutathione-Cu(I)-bathocuproine complex had to be introduced to account for the experimental observations. Analysis of the potential interference of the ternary complex with spectroscopic measurements of 1:2 Cu(I)-bathocuproine complex suggested that such interference was unlikely. These experiments demonstrated that the method is applicable

for determination of conditional stability constants of Cu(I) complexes with small peptides in competitive equilibration experiments.

Determination of conditional stability constants of Cu(I)-phytochelatin complexes was performed through experiments in which Cu(I), bathocuproine (B) and phytochelatin (P) were added incrementally to mixtures of the other two reagents. The experimental results were modeled using FITEQL 4.0. The results of Cu(I) titrations of phytochelatin/bathocuproine and phytochelatin titrations of Cu(I)/bathocuproine were consistent and were explained by formation of 1:1 and 1:2 Cu(I)-phytochelatin complexes and a polynuclear mixed complex BCu_2P . The following conditional stability constant values were obtained:

$$\log K_{\text{CuP}}^{\text{cond}} = 13.8 \pm 0.3, \log \beta_{\text{CuP}_2}^{\text{cond}} = 18.3 \pm 0.3, \log \beta_{\text{BCu}_2\text{P}}^{\text{cond}} = 27.8 \pm 0.3.$$

However, the results of bathocuproine titration experiments were inconsistent with those obtained in Cu(I) and phytochelatin titrations ($\log K_{\text{CuP}}^{\text{cond}} = 16.3$ and $\log \beta_{\text{CuP}_2}^{\text{cond}} = 21.2$). The discrepancy could be a result of slow kinetics of ligand exchange between a pre-formed Cu(I)-phytochelatin complexes and added bathocuproine and requires further investigation.

The sensitivity of the spectroscopic method does not allow it to be used at very low Cu(I) concentrations that would be relevant to natural waters. Formation of different species with different molar ratios of Cu(I) and phytochelatin is possible under such conditions.

The phytochelatin used in this study was a synthetic peptide with an α -linkage rather than the γ -linkage present in the naturally-produced peptide. These peptides have

been suggested to have similar metal binding properties; however, some differences may arise from the different structural arrangement of the natural and synthetic compounds. Certainly different metal-binding properties would be expected for the longer chain phytochelatins.

The determined conditional stability constants are very high. If present in natural waters, these complexes could influence copper speciation. However, both Cu(I) and phytochelatin are sensitive to oxidation and possibility of their survival under the oxic conditions is a balance between *in situ* production, uptake and degradation processes. These complexes are likely to form inside cells under reducing conditions and at high ligand concentrations. If present in open waters, such complexes could be expected to stabilize Cu(I) oxidation state and reduced form of phytochelatin.

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